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## ABSTRACT

We have previously implicated neuropilin-1 (NP1), a receptor for vascular endothelial growth factor (VEGF), in an autocrine survival pathway in breast carcinoma cells. Because NP1 promotes tumor cell survival, the goal of this proposal was to address the importance of NP1 in breast cancer progression. We have shown that NP1 is not preferentially expressed in highly aggressive and metastatic breast tumors. We have also demonstrated that the NP1 cytoplasmic domain is not required for its downstream signaling events. To explore the significance of NP1 on breast cancer progression *in vivo*, we attempted to use RNA interference to generate NP1-deficient cell lines. This strategy has been unsuccessful presumably due to a critical requirement for NP1 expression in survival of these cells. To circumvent this problem and to study signaling upstream of the NP1 receptor, we generated an  $\alpha 6 \beta 4$  integrin-deficient breast carcinoma cell line. Because  $\alpha 6 \beta 4$  regulates VEGF translation, the expression of VEGF is decreased in this cell line. Injection of this integrin-deficient cell line into mice produced significantly fewer orthotopic tumors compared to control cells because of increased apoptosis. We have demonstrated that this increased apoptosis correlates with decreased VEGF expression *in vivo* and presumably loss of NP1-mediated autocrine survival signaling.

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## **INTRODUCTION:**

The specific aims outlined in this proposal seek to address the importance of neuropilin-1 (NP1) in breast cancer progression. Importantly, our group has characterized a novel autocrine survival pathway in breast carcinoma cells specifically mediated by binding of vascular endothelial growth factor (VEGF) to NP1<sup>1</sup>. These studies were the first to identify a functional role for NP1 in tumor cells and they led us to hypothesize that NP1 plays a critical role in breast cancer metastasis. To further investigate the role of NP1 in breast cancer progression, we proposed to evaluate the expression of NP1 as a function of tumor grade, to determine the contribution of NP1 in tumor formation and metastasis, and to assess the importance of the NP1 cytoplasmic domain in downstream signaling events. The results of these experiments will determine whether NP1 is a critical mediator in the progression of metastatic breast cancer.

## **BODY:**

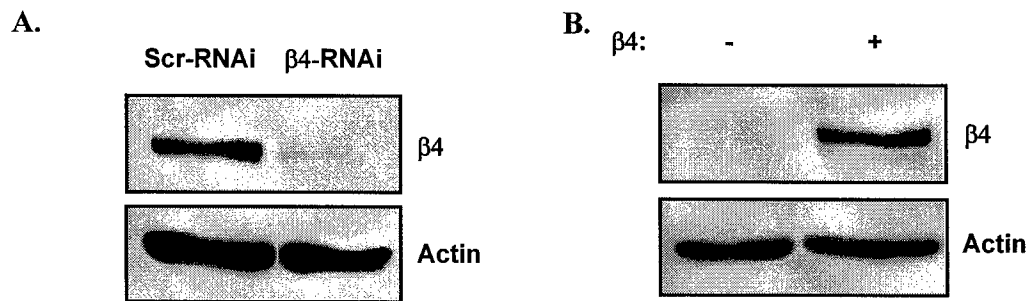
**Specific Aim #1: Investigate the expression of NP1 as a function of breast cancer progression.**

**Summary:** Because NP1 is expressed in highly aggressive and metastatic but not in less aggressive and non-metastatic breast carcinoma cell lines<sup>2</sup>, we wanted to investigate the expression of NP1 in human tumors as a function of breast cancer progression. In collaboration with Dr. Larry Brown, a clinical pathologist at BIDMC, we performed *in situ* hybridization experiments on greater than ten human cases at varying disease stages. We found that NP1 was expressed at a low level in tumor cells but that the level of NP1 expression did not increase with disease progression (data not shown). In addition, we found that NP1 was ubiquitously expressed at a high level in endothelial cells and fibroblasts. Although the expression of NP1 by endothelial cells was anticipated<sup>2</sup>, the presence of NP1 in fibroblasts was a novel finding. Interestingly, the expression of NP1 was recently reported in prostatic stromal cells corroborating our finding that NP1 is present in fibroblasts<sup>3</sup>. To date, we have been unable to confirm at the protein level the expression profile of NP1 in breast tumor progression or the expression of NP1 on stromal fibroblasts due to the lack of suitable antibodies to detect NP1 in paraformaldehyde-fixed human breast tissues. However, the future use of frozen tissue specimens to detect NP1 protein, as was recently reported in a study of colon adenocarcinomas, may overcome this limitation<sup>4</sup>.

**Specific Aim #2: Demonstrate that NP1 is sufficient and necessary for the progression of tumorigenic breast carcinoma cells to the metastatic phenotype.**

**Summary:** To explore the significance of NP1 on metastasis *in vivo*, we generated MDA-MB-231 and SUM-159 breast carcinoma cell lines that retrovirally expressed shRNA oligonucleotides specific to NP1 or control scrambled-sequence oligonucleotides. We found that we were unable to generate NP1-deficient cell lines using this RNA interference (RNAi) strategy even though the selected oligonucleotide sequence was efficient at reducing NP1 expression in transient transfection experiments. Consistent with its role as a pro-survival gene<sup>1</sup>, we concluded that NP1 is essential to the survival of these cells and that cells with stably decreased NP1 expression likely do not survive the selection process but instead undergo apoptosis. To circumvent this problem and to investigate the importance of NP1 in metastasis, a conditional system for shRNA expression will need to be generated for use in future studies.

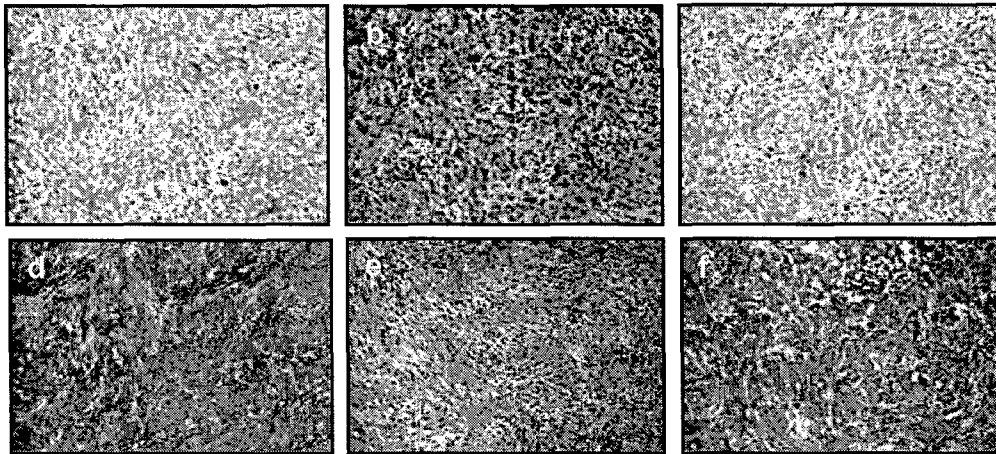
We initially attempted to stably reduce NP1 expression in the MDA-MB-231 breast carcinoma cell line. Because this approach was unsuccessful and to ensure that this phenomenon was not cell line specific, we next tried to decrease NP1 expression in the highly invasive SUM-159 breast carcinoma cell line<sup>5,6</sup>. Although this endeavor also failed to generate a NP1-deficient breast carcinoma cell line, we have found the SUM-159 cell line to be an excellent model system for investigating signaling pathways *in vitro* as well as for studying tumor formation and metastasis *in vivo*. Given that we have shown that the  $\alpha 6 \beta 4$  integrin regulates VEGF translation and that NP1 mediates tumor cell survival in a VEGF-dependent manner<sup>1,7</sup>, we generated a SUM-159 cell line that lacks expression of the  $\alpha 6 \beta 4$  integrin using shRNA retroviral strategies (Figure 1). The data generated with this  $\alpha 6 \beta 4$ -deficient cell line are summarized below.



**Figure 1. Generation of a  $\beta 4$ -deficient SUM-159 breast carcinoma cell line.** (A) SUM-159 cells were stably infected with a retrovirus expressing shRNA oligonucleotides to the  $\beta 4$  sequence ( $\beta 4$ -RNAi) or as control scrambled-sequence oligonucleotides (Scr-RNAi). Note the almost undetectable level of  $\beta 4$  protein expression in the  $\beta 4$ -deficient cell line as compared Scr-RNAi cell line. (B) To reintroduce expression of the  $\beta 4$ -subunit into the  $\beta 4$ -RNAi SUM-159 cell line, a  $\beta 4$  retrovirus was used to infect  $\beta 4$ -RNAi cells (-) yielding a  $\beta 4$ -RNAi+ $\beta 4$  cell line (+). In both A and B, protein lysates (30  $\mu$ g) were resolved by SDS-PAGE and immunoblotted for  $\beta 4$  and  $\beta$ -actin.

**Table 1. Expression of the  $\alpha 6 \beta 4$  integrin is necessary for tumor formation *in vivo*.** Scr-,  $\beta 4$ -RNAi, and  $\beta 4$ -RNAi+ $\beta 4$  SUM-159 cells ( $2 \times 10^6$  cells/injection) were injected into the mammary fat pad of immunocompromised mice. After 7-10 weeks, the frequency of tumor formation was reduced in mice injected with  $\beta 4$ -RNAi cells by 48% and 24% compared to Scr-RNAi and  $\beta 4$ -RNAi+ $\beta 4$  cells, respectively. The mean tumor weight in grams (g, +/-SEM) of the four mice bearing  $\beta 4$ -RNAi tumors was significantly less than the average weight of the Scr-RNAi tumors (two-tailed *t* test,  $p < 0.005$ ), whereas the  $\beta 4$ -RNAi+ $\beta 4$  tumors were comparable in weight to the Scr-RNAi tumors.

Cells	Number Mice	Number Tumors	Tumor Formation %	Tumor Weight (g)
Scr-RNAi	24	19	79	0.48 +/- 0.44
$\beta 4$ -RNAi	13	4	31	0.18 +/- 0.15
$\beta 4$ -RNAi+ $\beta 4$	11	6	55	0.55 +/- 0.72



**Figure 2. Increased apoptosis and decreased VEGF in mammary tumors formed in  $\beta 4$ -RNAi injected mice.** Assessment of the level of cell death (panels a-c) occurring in the mammary tumors that formed following orthotopic injection of the Scr-RNAi (a),  $\beta 4$ -RNAi (b) and  $\beta 4$ -RNAi+ $\beta 4$  (c) cell lines was determined by TUNEL labeling. The red-brown staining is indicative of apoptosis and is dramatically enhanced in the tumor formed following injection of the  $\beta 4$ -RNAi cell line (b) compared to the Scr-RNAi and  $\beta 4$ -RNAi+ $\beta 4$  cell lines (a and c, respectively). VEGF immunohistochemistry (panels d-f) performed on tumors resulting from injection of Scr-RNAi (d),  $\beta 4$ -RNAi (e) and  $\beta 4$ -RNAi+ $\beta 4$  (f) cell lines revealed a much higher level of VEGF expression in the Scr-RNAi (d) and  $\beta 4$ -RNAi+ $\beta 4$  (f) tumors compared to the  $\beta 4$ -RNAi (e) tumor.

**Specific Aim #3: Define the downstream signaling events mediated by NP1 that are responsible for metastasis.**

**Summary:** Our group has previously demonstrated that expression of NP1 in a non-NP1 expressing breast carcinoma cell line (MDA-MB-453 cells) is sufficient to promote survival of these cells in hypoxic conditions (5% CO<sub>2</sub>, 3% O<sub>2</sub>, 92 % N<sub>2</sub>)<sup>1</sup>. To investigate the involvement of the NP1 cytoplasmic-domain in this survival, MDA-MB-453 cells were transfected with full-length NP1, truncated NP1, or a control empty vector. After 72 hours in serum-free and hypoxic conditions, full-length NP1 decreased the level of apoptosis by 30% compared to cells transfected with a control empty vector. Unfortunately, the level of cell death was comparable for cells transfected with either full-length or truncated NP1. The more recently identified role of NP1 as a chemotaxis inhibitor prompted us to investigate whether the cytoplasmic domain of NP1 is important for its migratory function<sup>8</sup>. In three independent migration assays, the level of migration was comparable for breast carcinoma cells transfected with either full-length or truncated NP1. Taken together, these data suggest that the cytoplasmic domain of NP1 does not play a critical role in either its pro-survival or migration-inhibitor function.

The cytoplasmic domain of NP1 has been shown to interact with GIPC, a PDZ domain-containing protein in olfactory bulb membrane extracts<sup>9</sup>. To address whether this interaction is important in the signaling events mediated by NP1 in breast carcinoma cells, we attempted to co-immunoprecipitate endogenously expressed NP1 and GIPC in MDA-MB-231 cells. Using the same lysis buffer as described by Cai and Reed<sup>9</sup>, we were unable to co-immunoprecipitate NP1 and GIPC in two separate experiments and we do not currently believe that a NP1 and GIPC protein-protein interaction exists in breast carcinoma cells.

### **KEY RESEARCH ACCOMPLISHMENTS:**

- Generated a FLAG-epitope tagged human NP1 construct
- Demonstrated expression of NP1 at the mRNA level on stromal fibroblasts in human breast tumors
- Implemented a system for retroviral generation and propagation
- Developed a reliable strategy for utilizing RNAi in transient (oligonucleotide transfections) and stable (retrovirus-mediated) systems
- Generated a SUM-159 breast carcinoma cell line that is stably deficient for  $\alpha 6\beta 4$  expression by using RNAi technology and re-expressed  $\beta 4$  into this cell line producing a 'rescue' cell line to confirm functional specificity (Fig. 1)
- Established that loss of  $\alpha 6\beta 4$  expression enhances cell death in three-dimensional matrix assays
- Determined that re-expression of  $\beta 4$  into the  $\beta 4$ -deficient cell line and addition of exogenous VEGF both rescue this cell line from apoptosis
- Provided *in vivo* data implicating decreased  $\alpha 6\beta 4$  expression in inhibition of primary tumor formation and growth (Table 1)
- Showed that  $\beta 4$ -RNAi induced mammary tumors have elevated apoptosis and decreased VEGF expression (Fig. 2)
- Demonstrated that the  $\alpha 6\beta 4$  integrin influences the invasion of breast carcinoma cells independent of the Met growth factor receptor {Chung et al., 2004, APPENDIX}
- Substantiated the importance of the  $\alpha 6\beta 4$  integrin in invasion and migration of breast carcinoma cells using RNAi in *in vitro* assays {Lipscomb et al., 2003, APPENDIX}
- Identified NP1 as a regulator of chemotaxis in breast carcinoma cells {Bachelder et al., 2003, APPENDIX}
- Demonstrated a direct link between  $\alpha 6\beta 4$  expression and 4E-BP1 phosphorylation, VEGF expression, and survival of breast carcinoma cells {Chung et al., 2002, APPENDIX}

### **REPORTABLE OUTCOMES:**

#### **Published Manuscripts:**

S.O. Yoon, S. Shin, **E.A. Lipscomb**, and A.M. Mercurio. A novel mechanism for integrin-mediated Ras activation in breast carcinoma cells: The  $\alpha 6\beta 4$  integrin regulates erbB2 translation and transactivates EGFR/erbB2 signaling. (*Submitted*)

**E.A. Lipscomb**, K.J. Simpson, S.R. Lyle, J.E. Ring, A.S. Dugan, and A.M. Mercurio. The  $\alpha 6\beta 4$  integrin maintains the *in vivo* survival of human breast carcinoma cells. (*Submitted*)

**E.A. Lipscomb** and A.M. Mercurio. Mobilization and activation of a signaling competent  $\alpha 6\beta 4$  integrin underlies its contribution to carcinoma progression. (*In Press, Cancer Metastasis Rev.*)

J. Chung, S. Yoon, **E.A. Lipscomb**, and A.M. Mercurio. The Met receptor and  $\alpha 6\beta 4$  integrin function independently to promote carcinoma invasion. *J. Biol. Chem.* 279, 32287-32293, 2004.



**E.A. Lipscomb**, A.S. Dugan, I. Rabinovitz, and A.M. Mercurio. Use of RNA interference to inhibit integrin ( $\alpha 6 \beta 4$ )-mediated invasion and migration of breast carcinoma cells. *Clin. Exp. Metastasis* 20, 569-576, 2003.

R.E. Bachelder, **E.A. Lipscomb**, X. Lin, M.A. Wendt, N.H. Chadborn, B.J. Eickholt, and A.M. Mercurio. Autocrine regulation of carcinoma migration: A novel Semaphorin3A/NP1 inhibitory pathway is suppressed by VEGF. *Cancer Res.* 63, 5230-5233, 2003.

J. Chung, R.E. Bachelder, **E.A. Lipscomb**, L.M. Shaw, and A.M. Mercurio. Integrin ( $\alpha 6 \beta 4$ ) regulation of eIF-4E activity and VEGF translation: A survival mechanism for carcinoma cells. *J. Cell Biol.* 158, 165-174, 2002.

**Published Meeting Abstracts:**

**E.A. Lipscomb**, K.J. Simpson, S.R. Lyle, J.E. Ring, A.S. Dugan, and A.M. Mercurio. The  $\alpha 6 \beta 4$  integrin promotes tumor formation by regulating cell survival. *Keystone Symposium: The Role of Microenvironment in Tumor Induction and Progression*, 2005.

**E.A. Lipscomb**, K.J. Simpson, S.R. Lyle, J.E. Ring, A.S. Dugan, and A.M. Mercurio. The  $\alpha 6 \beta 4$  integrin promotes tumor formation by regulating cell survival. *Amer. Soc. Cell Biol.* 342, 2004.

**Personnel Supported from the Research Effort:**

Elizabeth Lipscomb, Ph.D.

**Seminar Presentations:**

"The  $\alpha 6 \beta 4$  integrin and tumor formation in mice". Division of Cancer Biology and Angiogenesis Data Club, Beth Israel Deaconess Medical Center, Boston, MA 02215. (October 2004)

"The  $\alpha 6 \beta 4$  integrin and tumor formation in mice". Division of Cancer Biology and Angiogenesis Data Club, Beth Israel Deaconess Medical Center, Boston, MA 02215. (November 2003)

"Use of RNA interference to inhibit integrin ( $\alpha 6 \beta 4$ )-mediated invasion and migration of breast carcinoma cells". Division of Cancer Biology and Angiogenesis Data Club, Beth Israel Deaconess Medical Center, Boston, MA 02215. (November 2002)

**Retrovirus Repository and Cell Lines:**

Retroviruses were generated using the RetroMax System (Imgenex) and stable virus-producing cell lines were generated following viral stock infection and drug-resistant selection.

Retroviral Stocks

Neuropilin1-pCLXSN  
GFP-pCLXSN

Cell Lines Infected/Selected

MDA-MB-453  
MDA-MB-453; MDA-MB-435

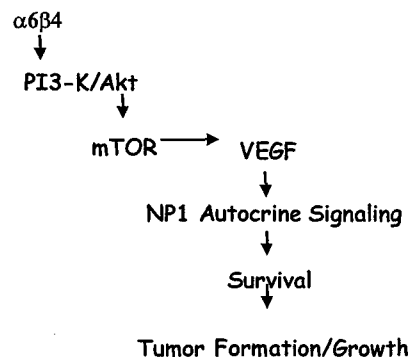
Empty-pCLXSN	MDA-MB-435
$\beta 4$ -pCLXSN	MDA-MB-435
	$\beta 4$ -RNAi SUM-159; $\beta 4$ -RNAi MDA-MB-231
Neuropilin1-pSuper.Retro (RNAi)	MDA-MB-231; SUM-159
SCRNeuropilin1-pSuper.Retro (RNAi)	MDA-MB-231; SUM-159
$\beta 4$ -pSuper.Retro (RNAi)	MDA-MB-231; SUM-159
SCR $\beta 4$ -pSuper.Retro (RNAi)	MDA-MB-231; SUM-159
Sema3A-pSuper.Retro (RNAi)	MDA-MB-435
SCRSema3A-pSuper.Retro (RNAi)	MDA-MB-435
Empty-pSuper.Retro (RNAi)	MDA-MB-435

### **Employment Opportunities:**

Based on experience and training supported by this award, I have been invited to interview for a position as an independent investigator at five universities and I have been offered two positions. At the present time, I am still considering my options but hope to end my search in the near future and begin my journey as a junior-faculty member in the Fall of 2005.

### **CONCLUSIONS:**

The goal of this proposal was to define the mechanism by which NP1 contributes to the survival and metastasis of breast carcinoma cells. We have determined that the level of NP1 does not increase in human tumors as a function of breast cancer progression (Aim 1). We have also shown that the cytoplasmic domain of NP1 is not required for its survival function or for its role as a chemotaxis inhibitor (Aim 3). To evaluate the importance of NP1 in tumor formation and metastasis, we attempted to generate NP1-deficient breast carcinoma cell lines using RNAi (Aim 2). Unfortunately, this approach has been unsuccessful likely because NP1 expression is essential to the survival of these cells. As an alternative approach, we generated a SUM-159 breast carcinoma cell line that lacks expression of the  $\alpha 6\beta 4$  integrin using the RNAi strategy and model system initially designed to study NP1 function. Given that the  $\alpha 6\beta 4$  integrin regulates VEGF translation and that NP1 mediates tumor cell survival in a VEGF-dependent pathway<sup>1,7</sup>, this approach enabled us to further study the signaling pathways upstream of the NP1 receptor. We have found that the  $\alpha 6\beta 4$  integrin regulates the translation of VEGF in a PI3-K- and mTOR-dependent pathway (Figure 3)<sup>7</sup>. In addition, we have shown that one mechanism by which VEGF promotes tumor cell survival is by activating the NP1 receptor<sup>1</sup>. Furthermore, we have shown that this  $\alpha 6\beta 4$ -mediated expression of VEGF and consequent tumor cell survival promotes tumor formation and growth *in vivo*.



**Figure 3.  $\alpha 6\beta 4$  contributes to tumor formation and growth by mediating tumor cell survival by regulating VEGF expression and signaling in a NP1-dependent pathway.**

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**REFERENCES:**

1. Bachelder, R.E. *et al. Cancer Res.* **61**, 5736-5740 (2001).
2. Soker, S., Takashima, S., Miao, H.Q., Neufeld, G., Klagsbrun, M. *Cell* **92**, 735-745 (1998).
3. Vanveldhuizen, P.J. *et al. Oncol. Rep.* **10**, 1067-1071 (2003).
4. Parikh, A.A. *et al. Am. J. Pathol.* **164**, 2139-2151 (2004).
5. Flanagan, L., Van Weelden, K., Ammerman, C., Ethier, S.P., Welsh, J. *Breast Cancer Res. Treat.* **58**, 193-204 (1999).
6. Zajchowski, D.A. *et al. Cancer Res.* **61**, 5168-5178 (2001).
7. Chung, J., Bachelder, R.E., Lipscomb, E.A., Shaw, L.M., Mercurio, A.M. *J. Cell Biol.* **158**, 165-174 (2002).
8. Bachelder, R.E. *et al. Cancer Res.* **63**, 5230-5233 (2003).
9. Cai, H. and Reed, R.R. *J. Neurosci.* **19**, 6519-6527 (1999).

**APPENDICES:**

J. Chung, S. Yoon, **E.A. Lipscomb**, and A.M. Mercurio. The Met receptor and  $\alpha 6 \beta 4$  integrin function independently to promote carcinoma invasion. *J. Biol. Chem.* **279**, 32287-32293, 2004.

**E.A. Lipscomb**, A.S. Dugan, I. Rabinovitz, and A.M. Mercurio. Use of RNA interference to inhibit integrin ( $\alpha 6 \beta 4$ )-mediated invasion and migration of breast carcinoma cells. *Clin. Exp. Metastasis* **20**, 569-576, 2003.

R.E. Bachelder, **E.A. Lipscomb**, X. Lin, M.A. Wendt, N.H. Chadborn, B.J. Eickholt, and A.M. Mercurio. Autocrine regulation of carcinoma migration: A novel Semaphorin3A/Neuropilin-1 inhibitory pathway is suppressed by VEGF. *Cancer Res.* **63**, 5230-5233, 2003.

J. Chung, R.E. Bachelder, **E.A. Lipscomb**, L.M. Shaw, and A.M. Mercurio. Integrin ( $\alpha 6\beta 4$ ) regulation of eIF-4E activity and VEGF translation: A survival mechanism for carcinoma cells. *J. Cell Biol.* 158, 165-174, 2002.

## The Met Receptor and $\alpha_6\beta_4$ Integrin Can Function Independently to Promote Carcinoma Invasion\*

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It has been proposed that a constitutive, physical association of the Met receptor and the  $\alpha_6\beta_4$  integrin exists on the surface of invasive carcinoma cells and that hepatocyte growth factor (HGF)-mediated invasion is dependent on  $\alpha_6\beta_4$  (Trusolino, L., Bertotti, A., and Comoglio, P. M. (2001) *Cell* 107, 643–654). The potential significance of these results prompted us to re-examine this hypothesis. Using three different carcinoma cell lines that express both Met and  $\alpha_6\beta_4$ , we were unable to detect the constitutive association of these receptors by co-immunoprecipitation. Moreover, carcinoma cells that lacked expression of  $\alpha_6\beta_4$  exhibited Met-dependent invasion toward HGF, and increasing Met expression by viral infection of these cells enhanced invasion without inducing  $\alpha_6\beta_4$  expression. Although expression of  $\alpha_6\beta_4$  in such cells enhanced their invasion to HGF, it also enhanced their ability to invade toward other chemoattractants such as lysophosphatidic acid, and this latter invasion was not inhibited by a function-blocking Met antibody. Finally, depletion of  $\beta_4$  by RNA interference in invasive carcinoma cells that express both receptors reduced the ability of these cells to invade toward HGF by ~25%, but it did not abrogate their invasion. These data argue that the invasive function of Met can be independent of  $\alpha_6\beta_4$  and that  $\alpha_6\beta_4$  has a generic influence on the invasion of carcinoma cells that is not specific to Met.

Understanding the receptor-mediated mechanisms that underlie invasive carcinoma is a timely and significant endeavor. The involvement of specific integrins and growth factor receptors in the invasive process is established, and several lines of evidence indicate that these two classes of surface receptors may cooperate to effect a wide range of biological functions, including the migration and invasion of tumor cells (2–4). The available data indicate that integrin and growth factor signaling can be synergistic, and in some cases physical association may occur between these receptor types. Insight into the nature of such receptor interactions has important implications not only for understanding the biology of tumor invasion but also for the design and use of therapeutics targeted to these receptors (5).

An integrin of particular relevance to invasive carcinoma is  $\alpha_6\beta_4$  (6–8). This integrin, which is expressed primarily on the basal surface of most epithelia and in most carcinoma cells, is defined as an adhesion receptor for most of the known basement membrane laminins (6, 9). A primary function of  $\alpha_6\beta_4$ , revealed by studies of knock-out mice, is to maintain the integrity of epithelia (10, 11). This critical role for  $\alpha_6\beta_4$  derives from its ability to mediate the formation of stable adhesive structures, termed hemidesmosomes, on the basal cell surface that link the cytokeratin network with laminins in the basement membrane (12). Recent studies have revealed novel and important functions for this integrin in the migration and invasion of carcinoma cells (13). The expression of  $\alpha_6\beta_4$  is maintained or often increased in invasive and metastatic carcinomas, and its expression level correlates with the progression of these carcinomas (14). More recently, compelling data were reported that suggest the  $\alpha_6\beta_4$  integrin is essential for squamous carcinogenesis (15).

Given the potential importance of the  $\alpha_6\beta_4$  integrin to invasive carcinoma, extensive efforts are being made to define the mechanisms by which it facilitates the invasive process. Advances include the observation that  $\alpha_6\beta_4$  is localized to the leading edge of migrating carcinoma cells where it can contribute to the formation and stabilization of actin protrusions (16, 17). In addition, there is evidence from several laboratories indicating that  $\alpha_6\beta_4$  stimulates the activity of phosphoinositide 3-OH kinase (PI3K)<sup>1</sup> in invasive carcinoma cells and that PI3K is essential for migration and invasion (7, 18). Interestingly, it has been suggested that  $\alpha_6\beta_4$  activates PI3K and mediates invasion through its ability to cooperate with specific growth factor receptors (1, 4, 18). For example,  $\alpha_6\beta_4$  has been shown to associate with erbB2 on the surface of breast carcinoma cells, and this interaction appears to facilitate activation of PI3K and invasion (18, 19).

More recently, it was argued that  $\alpha_6\beta_4$  functions as an essential adaptor protein for the Met receptor in invasive carcinoma cells (1). The impact of this finding is amplified by the fact that substantial evidence exists for the importance of Met in the scattering, invasion, and metastasis of tumor cells (20, 21). If  $\alpha_6\beta_4$  were an essential, specific adaptor for Met function in these events, the consequences for carcinoma biology and therapy would be profound. The potential significance of these results prompted us to re-examine the central findings of this study, which were that a selective physical association between Met and  $\alpha_6\beta_4$  exists on the surface of invasive carcinoma cells and that Met cannot promote invasion in the absence of  $\alpha_6\beta_4$  expression.

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<sup>1</sup> The abbreviations used are: PI3K, phosphoinositide 3-OH kinase; Ab, antibody; mAb, monoclonal antibody; siRNA, small interfering RNA; HGF, hepatocyte growth factor; EGF, epidermal growth factor; SCR, scrambled.

## EXPERIMENTAL PROCEDURES

**Cells**—MDA-MB-231 and MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University (Washington, DC), and A431 cells were purchased from American Type Culture Collection. Cells were grown in low glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 25 mM Hepes. The generation of stable transfectants of MDA-MB-435 cells that express the  $\alpha_6\beta_4$  integrin has been described previously (7). For Met expression studies, a vesicular stomatitis virus-coated retrovirus containing Met cDNA was obtained from Dr. Morag Park (McGill University, Montreal, Quebec, Canada). SUM-159 cells were obtained from Dr. Stephen Ethier at the University of Michigan Comprehensive Cancer Center (Ann Arbor, MI) and maintained in Ham's F-12 medium containing 5% fetal bovine serum, 5  $\mu$ g/ml insulin, 1 mg/ml hydrocortisol, 1% penicillin-streptomycin, and 25 mM Hepes (22).

To create  $\beta_4$  siRNA-pSUPER and  $\beta_4$  SCR-pSUPER expression vectors, the following oligonucleotides (Invitrogen) were annealed and ligated into pSUPER (a gift from R. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands) between the BglII and HindIII sites:  $\beta_4$  siRNA, 5'-gateccccGAGCTGCACGAGTGTGTCTtaagagaGACACTCCGTCGAGCTCTtttttggaaa-3' and 5'-agcttttccaaaaGAGCTGCACGAGTGTGTCTctcttgaaGACACTCCGTCGAGCTCggg-3';  $\beta_4$  SCR, 5'-gateccccGTTGTGCGAGGAACGGTGCcttaagagaGGCACCGTTCCTCGACAACTtttttggaaa-3' and 5'-agcttttccaaaaGTTGTGCGAGGAACGGTGCctcttgaaGGCACCGTTCCTCGACAACTggg-3'. EcoRI- and XhoI-digested inserts containing the H1-RNA promoter and either  $\beta_4$  siRNA or  $\beta_4$  SCR targeting oligonucleotides from pSUPER were then subcloned into pSUPER.retro (Oligoengine, Seattle, WA) to generate  $\beta_4$  siRNA and  $\beta_4$  SCR-pSUPER.retro. All plasmids were sequenced to confirm that they were correct.

To generate retroviruses,  $\beta_4$  siRNA or  $\beta_4$  SCR-pSUPER.retro and expression plasmids containing envelope and packaging proteins required for viral propagation (Imgenex, San Diego, CA) were transfected into 293T cells ( $3 \times 10^6$  cells/100-mm plate) with LipofectAMINE (Invitrogen) as described by the manufacturer. Three days after transfection, viral supernatants were harvested, and SUM-159 recipient cells were infected in the presence of a serum-containing medium supplemented with 8  $\mu$ g/ml polybrene (Sigma). Following infection for 24 h, resistant cells were selected with 4.0  $\mu$ g/ml puromycin (Clontech), and cell lines that stably expressed  $\beta_4$  siRNA or  $\beta_4$  SCR were generated. The stable  $\beta_4$  siRNA SUM-159 cell line was then sorted by flow cytometry, and the population of cells that expressed the lowest level of  $\beta_4$  was isolated. The sorting and subsequent surface labeling analysis of the  $\beta_4$ -deficient cell line was performed with 3E1, a mouse anti- $\beta_4$  integrin antibody (Chemicon, Temecula, CA). The decreased level of  $\beta_4$  expression in this cell line was further confirmed by Western blotting with a rabbit polyclonal anti- $\beta_4$  integrin antibody (505) (17).

**Biochemical Analyses**—For the co-immunoprecipitation studies, cells were extracted for 15 min at 4 °C with a buffer containing 50 mM Hepes (pH 7.4), 5 mM EDTA, 2 mM EGTA, 150 mM NaCl, 10% glycerol, and 1% Nonidet P-40 in the presence of protease and phosphatase inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 mg/ml each of aprotinin, leupeptin, pepstatin, sodium fluoride, and sodium orthovanadate). Extracts were clarified by centrifugation at 13,000 rpm for 10 min, the supernatants were collected, and their protein concentrations were determined using the Bio-Rad DC protein assay kit. Immunoprecipitations were performed with equal amounts of total protein. Extracts were preabsorbed overnight using protein A- and G-Sepharose beads (Amersham Biosciences) to prevent nonspecific binding. After centrifugation at 2000 rpm for 5 min to pellet these beads, the supernatants were incubated overnight at 4 °C with 1  $\mu$ g of the following antibodies: rat  $\beta_4$  mAb, 439-9B (obtained from Rita Falcioni, Regina Elena Cancer Institute, Rome, Italy), polyclonal anti-human Met C-12 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse  $\beta_4$  mAb, 3E1 (Chemicon), mouse Met mAb DO-24 (Upstate Biotechnology, Lake Placid, NY), and the appropriate rat, rabbit, and mouse IgGs (Sigma). Immune complexes were precipitated with protein A- and G-Sepharose, washed four times with extraction buffer, and eluted in 1 $\times$  reducing sample buffer.

For immunoblotting, cell extracts were prepared as described previously. These extracts or the immune complexes were separated by SDS-PAGE and transferred to Hybond-C nitrocellulose membranes (Amersham Bioscience). Membranes were blocked for 1 h using 50 mM Tris buffer (pH 7.5) containing 0.15 M NaCl, 0.05% Tween 20 (TBS-T), and 5% Carnation dry milk. The membranes were incubated overnight in the same buffer containing antibodies specific for either the  $\beta_4$  integrin (505), anti- $\beta$ -actin (Sigma), or Met (C-12, Santa Cruz Biotech-

nology, or (DO-21, Upstate Biotechnology). After three 10-min washes in TBS-T, the membranes were incubated for 1 h in blocking buffer containing horseradish peroxidase-conjugated secondary antibodies. After three 10-min washes in TBS-T, proteins were detected by enhanced chemiluminescence (Pierce).

For immunoblots involving the phosphotyrosine Ab (anti-phosphotyrosine, clone PY99, Cell Signaling Technology, Beverly, MA), the membranes were blocked for 1 h using 10 mM Tris buffer (pH 7.5) containing 0.5 M NaCl, 0.1% Tween 20, and 2% bovine serum albumin (w/v). The membranes were washed briefly and then incubated overnight at 4 °C in the same blocking buffer containing the phosphotyrosine Ab. After washing, the filters were incubated for 1 h in blocking buffer containing horseradish peroxidase-conjugated secondary Ab, and the proteins were detected by enhanced chemiluminescence.

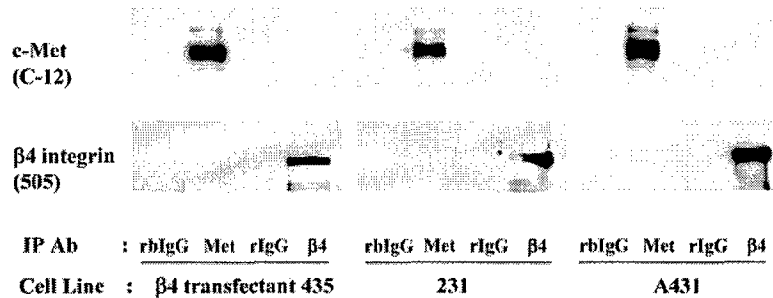
**Invasion Assays**—To prepare the Transwell membranes (Corning Glass) for the invasion assays, 0.5  $\mu$ g of Matrigel (Collaborative Biotech, Waltham, MA) was diluted with cold water and left to dry overnight onto the upper side of the membranes at 25 °C. On the following day, the lower sides of the membranes were coated with 10  $\mu$ g/ml laminin-1 (Calbiochem) and again left to dry overnight at 4 °C. The membranes were blocked with Dulbecco's modified Eagle's medium (or Ham's F-12 medium for SUM-159) for 1 h at 37 °C. Cells were trypsinized on the following day and resuspended in Dulbecco's modified Eagle's medium or Ham's F-12 medium containing 0.25% heat-inactivated lipid-free bovine serum albumin, and a total of  $5 \times 10^4$  cells was added to the upper chamber of each well. Chemoattractants (50 ng/ml HGF (R & D System, Minneapolis, MN), 100 ng/ml lysophosphatidic acid (Sigma), or 3T3 cell-conditioned medium) were added to the bottom wells in Dulbecco's modified Eagle's medium-bovine serum albumin. In some assays, cells were preincubated with a function-blocking Met mAb, clone 95309 (R & D Systems), for 1 h at 4 °C before plating. After incubating for 2 or 4 h at 37 °C, non-migrating cells were mechanically removed from the upper chamber with a cotton swab. Cells that had migrated to the lower side of the Transwell membrane were fixed with 100% methanol and stained with 0.2% crystal violet in 2% ethanol. Invasion was quantified by counting the number of cells per square millimeter using a reticule with bright-field optics.

## RESULTS

**Lack of Evidence for Constitutive Association between Met and  $\alpha_6\beta_4$  Integrin in Invasive Carcinoma Cells**—Evidence to support a physical association between Met and the  $\alpha_6\beta_4$  integrin has been shown in co-immunoprecipitation experiments using COS cells transfected to express high levels of both Met and  $\alpha_6\beta_4$ , as well as in carcinoma cells that express both receptors endogenously (1). In particular, it was reported that  $\beta_4$  immunoprecipitates of A431 cells contain significant amounts of Met. To confirm these findings, we performed co-immunoprecipitation assays using A431 cells, as well as MDA-MB-231 breast carcinoma cells, which express high levels of both  $\alpha_6\beta_4$  and Met. In addition, MDA-MB-435 cells that stably express the  $\alpha_6\beta_4$  integrin were examined because they had been used previously to demonstrate the necessity of  $\alpha_6\beta_4$  for HGF-dependent invasion (1). To assess a putative physical association of these two receptors, cells were extracted with the same Nonidet P-40 (1%) buffer used previously (1), and extracts were "precleared" and then immunoprecipitated with Abs specific for either the  $\beta_4$  integrin subunit (439-9B) or Met (C-12). Subsequently, the precipitates were analyzed by immunoblotting with either  $\beta_4$ -specific polyclonal (505) Ab or Met Ab (C-12). As shown in Fig. 1A, the  $\beta_4$  integrin subunit was evident in the  $\beta_4$  immunoprecipitates, and Met was evident in the Met immunoprecipitates. However, there was no indication of Met in the  $\beta_4$  immunoprecipitates or of  $\beta_4$  in the Met immunoprecipitates. To exclude the possibility that the co-immunoprecipitation of these two receptors is dependent on the detergent used for extraction, we also used Triton X-100-based buffer (1%) but failed to detect evidence for their physical association (data not shown). In addition, other Abs were used for the co-immunoprecipitation studies in A431 cells. Two different Met Abs (C-12 and DO-24) immunoprecipitated Met effectively but not the  $\beta_4$  integrin subunit (Fig. 1B). Similarly,  $\beta_4$  integrin subunit Abs

A.

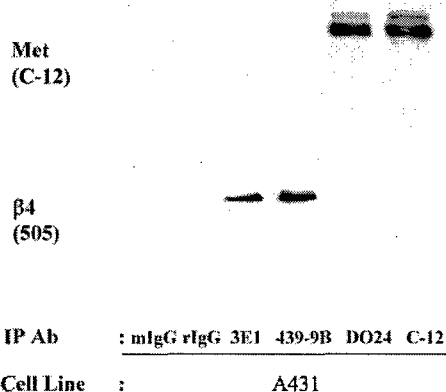
Blot:



**FIG. 1. Lack of evidence for a constitutive association between Met and the  $\alpha_6\beta_4$  integrin.** A, extracts from the indicated cell lines were immunoprecipitated (IP) with antibodies against Met (clone C-12),  $\beta_4$  integrin (clone 439-9B), or the indicated IgG control (rIgG, rabbit IgG; rIgG, rat IgG). Immunoblot analysis of these immunoprecipitates was performed using anti-Met (clone C-12) and anti- $\beta_4$  integrin polyclonal (505) antibodies. B, extracts from A431 cells were immunoprecipitated with  $\beta_4$  integrin-specific antibodies (mouse monoclonal clone DO-24 and rabbit polyclonal clone C-12) or IgG controls (mouse IgG and rat IgG), and immunoprecipitates were analyzed by immunoblotting as described in A.

B.

Blot:



(439-9B and 3E1) immunoprecipitated the  $\beta_4$  integrin subunit but not Met (Fig. 1B).

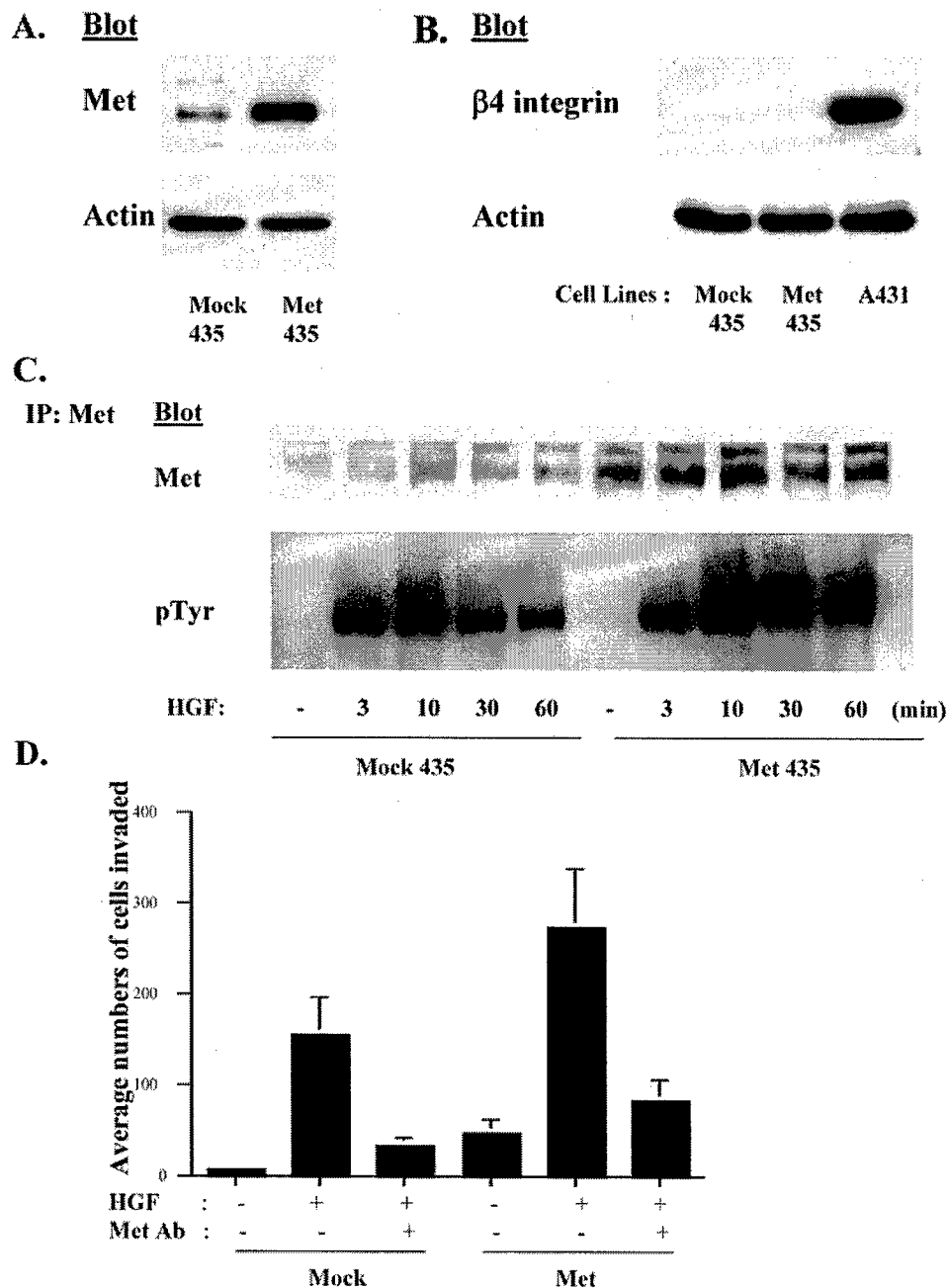
**Met Can Function Independently of the  $\alpha_6\beta_4$  Integrin in MDA-MB-435 Cells**—To evaluate the hypothesis that the invasive function of Met is dependent on the  $\alpha_6\beta_4$  integrin, we used MDA-MB-435 cells that express Met but not  $\alpha_6\beta_4$  (Fig. 2). In addition, the level of Met expression was increased in these cells by retroviral infection with a Met cDNA. This infection increased Met expression by ~2-fold (Fig. 2A), but it did not induce expression of  $\alpha_6\beta_4$  (Fig. 2B). The ability of Met to signal in the absence of  $\alpha_6\beta_4$  expression was examined. In the control cells, HGF stimulation induced a rapid and marked increase in tyrosine phosphorylation of Met as assessed by immunoprecipitating Met followed by immunoblotting these immunoprecipitates with a phosphotyrosine-specific Ab (Fig. 2C). The intensity of this phosphotyrosine signal was increased in the Met infectants, in agreement with the fact that these cells express significantly more Met than do the control infectants (Fig. 2A).

Subsequently, we assessed the ability of the control and Met infectants to invade toward HGF. Invasion assays were performed for 4 h without prior serum deprivation. As shown in Fig. 2D, HGF induced the invasion of the control cells significantly, and this invasion was abrogated by a function-blocking Met-specific antibody. A 2-fold induction of HGF-dependent invasion was observed in the Met infectants compared with control cells, substantiating the conclusion that Met promotes invasion in the absence of  $\alpha_6\beta_4$  integrin (Fig. 2D).

**$\alpha_6\beta_4$  Integrin Has a Generic Influence on Carcinoma Invasion That Is Not Specific to Met**—A key finding in the previous study, which concluded that  $\alpha_6\beta_4$  is necessary for the invasive function of Met, was that expression of  $\alpha_6\beta_4$  in MDA-MB-435

cells induces their ability to invade toward HGF (1). Although our results confirm the observation that expression of the  $\alpha_6\beta_4$  integrin increases the invasion of these cells toward HGF, we observed that the mock transfectants, which lack  $\alpha_6\beta_4$ , exhibited significant migration toward HGF (Fig. 3A). Moreover, the level of invasion induced by expression of  $\alpha_6\beta_4$  is comparable with the increased invasion that results from increased Met expression and that this latter mode of invasion occurs in the absence of  $\alpha_6\beta_4$  (Fig. 2D). Furthermore, expression of  $\alpha_6\beta_4$  in MDA-MB-435 cells also enhanced the ability of the MDA-MB-435 cells to invade toward other chemoattractants such as lysophosphatidic acid, and this invasion was not inhibited by a function-blocking Met antibody (Fig. 3B). This latter result indicated that the expression of  $\alpha_6\beta_4$  can enhance the ability of MDA-MB-435 cells to invade independently of Met expression. Invasion assays were performed for 4 h in serum-free medium, and, under these conditions, no significant increase in apoptosis was observed for any cell population as assessed by annexin-V fluorescein isothiocyanate staining (data not shown).

To assess the putative functional dependence of Met on the  $\alpha_6\beta_4$  integrin from a different perspective, we used SUM-159 cells (22). These invasive breast carcinoma cells express both  $\alpha_6\beta_4$  and Met (Fig. 4A). Using this cell line, we generated a SUM-159 cell line deficient in  $\beta_4$  integrin expression using siRNA strategies (23). Stable infectants that exhibited a reduction in  $\beta_4$  expression were sorted by fluorescence-activated cell sorter using a  $\beta_4$ -specific antibody, and a population of cells was isolated that exhibited no detectable  $\beta_4$  expression as evident by immunoblotting (Fig. 4A). Notably, the loss of  $\beta_4$  expression had no effect on Met expression in these cells (Fig. 4A). SUM-159 cells exhibited a robust invasion toward HGF



**FIG. 2. Met can function independently of the  $\alpha_6\beta_4$  integrin in MDA-MB-435 cells.** *A and B*, MDA-MB-435 mock and Met infectants and A431 cells were extracted, and equal amounts of protein extracts were analyzed by immunoblot analysis using Met (clone C-12),  $\beta_4$  (clone 505), and actin-specific antibodies. *C*, MDA-MB-435 mock and Met infectants were extracted at the designated time points during HGF treatment, and extracts were immunoprecipitated (IP) with anti-Met antibody (clone C-12). Immunoblotting was performed with either an anti-Met antibody (upper panel, clone C-12) or an anti-pTyr antibody (lower panel, clone p-Y-100). *D*, the ability of MDA-MB-435 mock and Met transfectants to invade Matrigel toward HGF was investigated in a 4-h assay. Prior to these assays, cells were incubated for 30 min with either a control IgG (–) or a Met-specific antibody (clone 95309). The mean number of invasive cells ( $\pm$  S.D.) from five independent fields/well is indicated on the y axis. Similar data were obtained in five separate experiments.

(Fig. 4B). Loss of  $\alpha_6\beta_4$  expression reduced the ability of these cells to invade toward HGF by  $\sim 25\%$ , but it did not abrogate their invasion (Fig. 4B). Moreover, loss of  $\alpha_6\beta_4$  expression also diminished the invasion of SUM-159 cells toward 3T3 cell-conditioned medium by  $\sim 30\%$ .

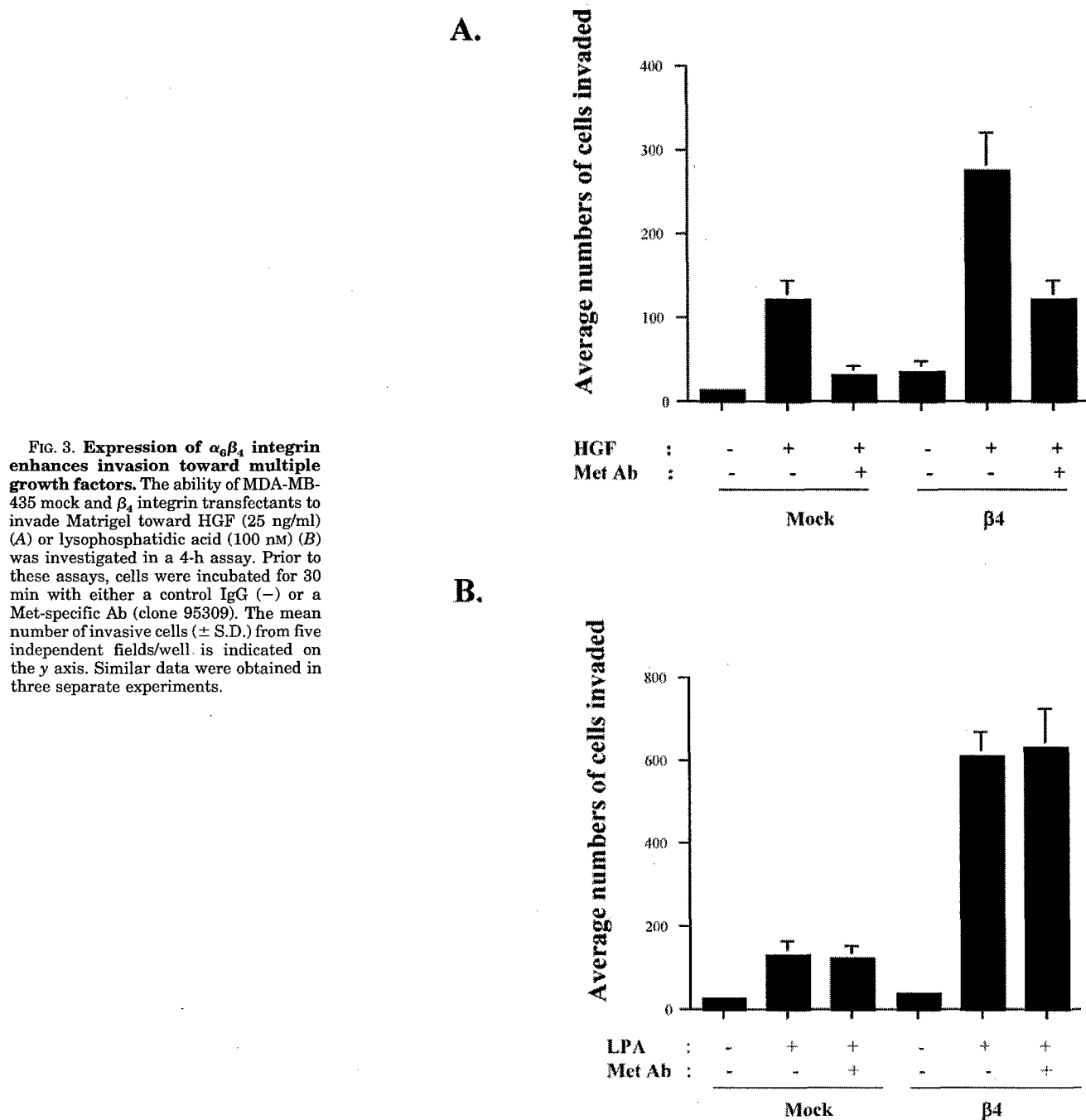
#### DISCUSSION

The report that the invasive function of the Met receptor is dependent on a physical association with the  $\alpha_6\beta_4$  integrin, which provides a “signaling adaptor function,” afforded a compelling model for invasive carcinoma that linked these two receptors (1).

The data obtained in our study unfortunately do not support the central tenets of this model. Rather, our data argue that the invasive function of Met can be independent of  $\alpha_6\beta_4$  and that  $\alpha_6\beta_4$  has a generic influence on the invasion of carcinoma cells that is not specific for HGF-dependent invasion.

The demonstration of a physical association between an integrin and a growth factor receptor provides *prima facie* evidence for cooperativeness of function. For this reason, the previous finding that Met and  $\alpha_6\beta_4$  could be co-immunoprecipitated from GTL-16 cells, which overexpress a constitutively active form of Met, from COS cells engineered to express both



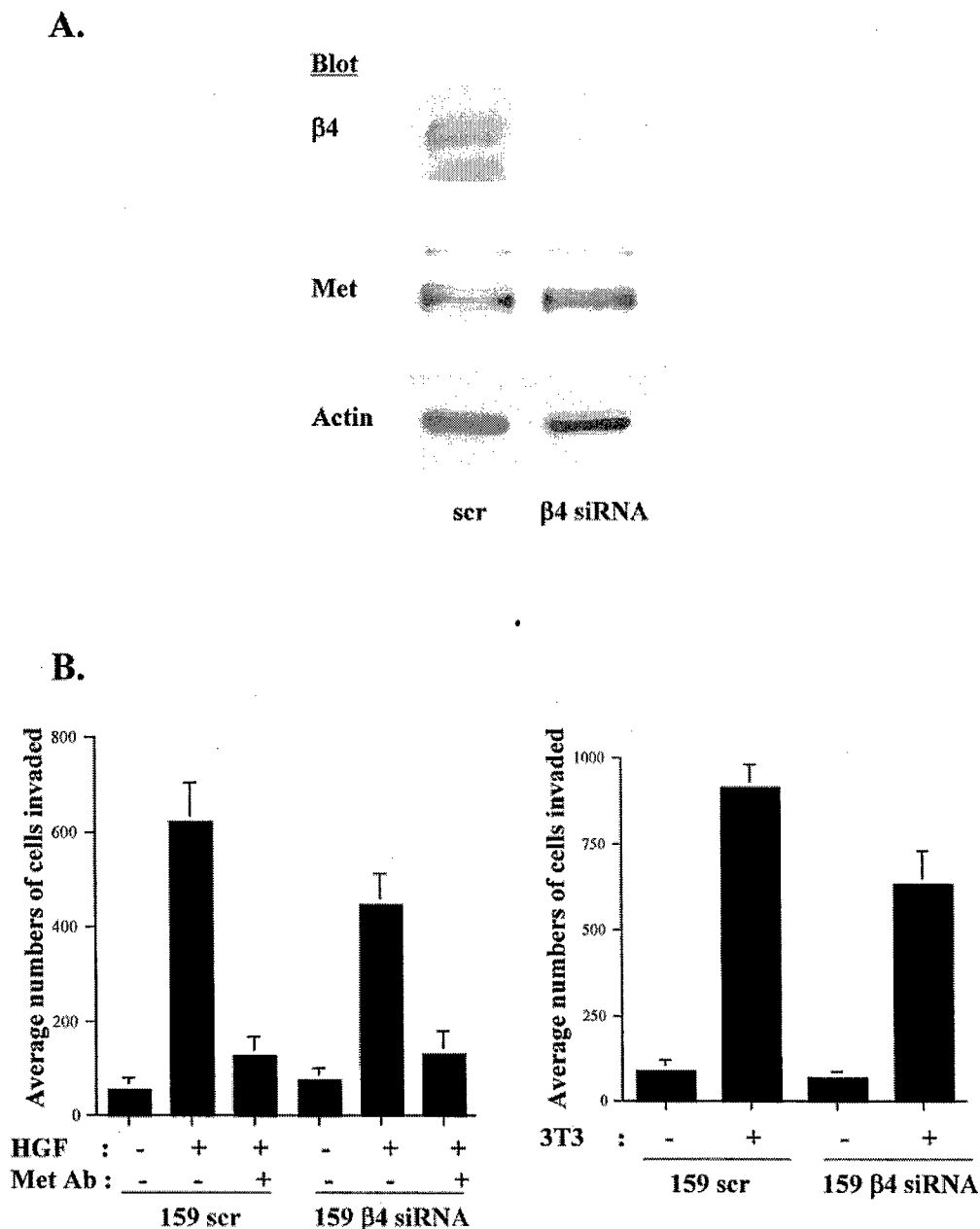


**FIG. 3. Expression of  $\alpha_6\beta_4$  integrin enhances invasion toward multiple growth factors.** The ability of MDA-MB-435 mock and  $\beta_4$  integrin transfectants to invade Matrigel toward HGF (25 ng/ml) (A) or lysophosphatidic acid (100 nM) (B) was investigated in a 4-h assay. Prior to these assays, cells were incubated for 30 min with either a control IgG (-) or a Met-specific Ab (clone 95309). The mean number of invasive cells ( $\pm$  S.D.) from five independent fields/well is indicated on the y axis. Similar data were obtained in three separate experiments.

receptors at high levels and from A431 cells, which also express both receptors, strengthened the possibility of a functional dependence (1). Despite using three different carcinoma cell lines that express both Met and  $\alpha_6\beta_4$  (A431, MDA-MB-231, and MDA-MB-435, engineered to express  $\alpha_6\beta_4$ ), however, we were unable to detect any evidence for the constitutive association of these receptors by co-immunoprecipitation. The reason for the difference between our results and those of Trusolino *et al.* (1) is unclear. It is worth noting, however, that in the previous study co-immunoprecipitation data were not provided for either MDA-MB-231 cells or MDA-MB-435/ $\beta_4$  cells, and an association between Met and  $\alpha_6\beta_4$  in A431 cells was detected only by immunoprecipitation with a  $\beta_4$  integrin antibody and immunoblotting with a Met Ab and not vice versa. In addition, the use of another purified  $\beta_4$  integrin Ab (439-9B) failed to co-immunoprecipitate Met. Although our data refute the existence

of a constitutive association of Met with  $\alpha_6\beta_4$  in carcinoma cells, they do not exclude the occurrence of a transient association between these two receptors in certain physiological situations or the possibility that a spurious association may occur on their gross overexpression.

The lack of evidence for a physical association between  $\alpha_6\beta_4$  and Met does not negate the possibility that they exhibit functional cooperativeness. To evaluate the hypothesis that the invasive function of Met depends on  $\alpha_6\beta_4$ , we assessed the invasion of MDA-MB-435 cells, which express Met but not  $\alpha_6\beta_4$ . These cells exhibited significant invasion toward HGF, and their rate of invasion increased in response to increasing Met expression by retroviral infection. The fact that these cells are capable of significant HGF-dependent invasion in the absence of  $\alpha_6\beta_4$  expression argues against the necessity of this integrin for Met function. Moreover, a key finding in the pre-



**FIG. 4. Loss of  $\alpha_6\beta_4$  integrin expression in SUM-159 cells reduces but does not abrogate invasion toward HGF and 3T3 cell-conditioned medium.** A, SUM-159 cells stably expressing either scrambled (scr) or  $\beta_4$  integrin RNAi ( $\beta_4$  siRNA) oligonucleotides were extracted, and equal amounts of protein extracts were analyzed by immunoblot analysis using Met (C-12),  $\beta_4$  (505), and actin-specific Abs. B, the ability of the cells described in A to invade Matrigel toward HGF (50 ng/ml) or 3T3 cell-conditioned medium was investigated in a 2-h assay. The mean number of invasive cells ( $\pm$  S.D.) from the five independent field per well is indicated on the y axis. Similar data were obtained in three separate experiments.

vious study was that expression of  $\alpha_6\beta_4$  in MDA-MB-435 cells induced their ability to invade toward HGF but not toward EGF. We note, however, EGF is not a suitable negative control because MDA-MB-435 cells lack expression of the EGF receptor (24).<sup>2</sup> Additional support for the hypothesis that the invasive function of Met can occur independently of  $\alpha_6\beta_4$  is provided by our data on SUM-159 breast carcinoma cells. These invasive cells express both Met and  $\alpha_6\beta_4$ , and they exhibit a robust invasion toward HGF. Our finding that elimination of  $\alpha_6\beta_4$  expression using a  $\beta_4$ -specific siRNA reduced but did not abrogate invasion toward HGF, however, argues against the conclusion that  $\alpha_6\beta_4$  is an essential adaptor for Met in promoting

carcinoma invasion. The strength of the SUM-159 data, in contrast to MDA-MB-435 cells, is that this cell line exhibits endogenous expression of both receptors, and the assumption can be made that if Met function were dependent on  $\alpha_6\beta_4$ , it should be evident in such a cell line.

Based on several studies as well as the findings reported here, a consensus is emerging that  $\alpha_6\beta_4$  cooperates with growth factor receptors to promote carcinoma invasion and other functions (7, 18, 25). Perhaps the most conclusive evidence in this regard is the finding that macrophage-stimulating protein on binding to its receptor, the Ron tyrosine kinase, promotes an association between Ron and  $\alpha_6\beta_4$  that results in PI3K activation and consequent migration (4). There is also evidence that  $\alpha_6\beta_4$  can cooperate with erbB2 in breast carcinoma cells to

<sup>2</sup> R. Bachelder, personal communication.

activate PI3K and promote invasion (18). The conclusion that the function of one specific growth factor receptor (Met) is absolutely dependent on  $\alpha_6\beta_4$  for promoting invasion, however, is not supported by our data. A more appropriate assessment of the relationship between Met and  $\alpha_6\beta_4$  would be that expression of  $\alpha_6\beta_4$  can enhance invasion toward several growth factors, including HGF and lysophosphatidic acid, as well as those present in 3T3 cell-conditioned medium (Figs. 3 and 4). At the same time, our results indicate that the ability of Met to promote invasion is not dependent on  $\alpha_6\beta_4$  in these cells and that increasing Met expression in the absence of  $\alpha_6\beta_4$  enhances HGF-mediated invasion.

The mechanism that underlies the ability of  $\alpha_6\beta_4$  to promote invasion likely involves its ability to stimulate PI3K. Compelling evidence exists for  $\alpha_6\beta_4$ -mediated activation of this enzyme by mechanisms that include phosphorylation of insulin receptor substrate proteins (26), cooperation with erbB2 (18, 19) and Ron (4), and the elaboration of vascular endothelial growth factor autocrine signaling (27). In addition, the regulated expression of specific transcription factors such as nuclear factor of activated T cells by  $\alpha_6\beta_4$  may contribute to the invasive phenotype (28). Clearly, Met is one of several growth factor receptors with a function that may be enhanced by  $\alpha_6\beta_4$  expression but that can signal and promote invasion in the absence of  $\alpha_6\beta_4$  expression. The challenge ahead is to define the mechanisms by which expression of  $\alpha_6\beta_4$  enhances the function of multiple growth factor receptors.

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#### REFERENCES

- Trusolino, L., Bertotti, A., and Comoglio, P. M. (2001) *Cell* **107**, 643–654
- Beviglia, L., and Kramer, R. H. (1999) *Int. J. Cancer* **83**, 640–649
- Stupack, D. G., and Cheresch, D. A. (2003) *Oncogene* **22**, 9022–9029
- Santoro, M. M., Gaudino, G., and Marchisio, P. C. (2003) *Dev. Cell* **5**, 257–271
- Tantivejkul, K., Vucenik, I., and Shamsuddin, A. M. (2003) *Anticancer Res.* **23**, 3681–3689
- Mercurio, A. M., and Rabinovitz, I. (2001) *Semin. Cancer Biol.* **11**, 129–141
- Shaw, L. M., Rabinovitz, I., Wang, H. H., Toker, A., and Mercurio, A. M. (1997) *Cell* **91**, 949–960
- Rabinovitz, I., and Mercurio, A. M. (1996) *Biochem. Cell Biol.* **74**, 811–821
- Mercurio, A. M., Bachelder, R. E., Chung, J., O'Connor, K. L., Rabinovitz, I., Shaw, L. M., and Tani, T. (2001) *J. Mammary Gland Biol. Neoplasia* **6**, 299–309
- Dowling, J., Yu, Q. C., and Fuchs, E. (1996) *J. Cell Biol.* **134**, 559–572
- Vandernout, R., Krimpenfort, P., Calafat, J., Niessen, C. M., and Sonnenberg, A. (1996) *Nat. Genet.* **13**, 366–369
- Borradori, L., and Sonnenberg, A. (1999) *J. Invest. Dermatol.* **112**, 411–418
- Mercurio, A. M., Rabinovitz, I., and Shaw, L. M. (2001) *Curr. Opin. Cell Biol.* **13**, 541–545
- Mercurio, A. M., Bachelder, R. E., Rabinovitz, I., O'Connor, K. L., Tani, T., and Shaw, L. M. (2001) *Surg. Oncol. Clin. N. Am.* **10**, 313–328 (Review viii–ix)
- Dajee, M., Lazarov, M., Zhang, J. Y., Cai, T., Green, C. L., Russell, A. J., Marinkovich, M. P., Tao, S., Lin, Q., Kubo, Y., and Khavari, P. A. (2003) *Nature* **421**, 639–643
- Rabinovitz, I., and Mercurio, A. M. (1997) *J. Cell Biol.* **139**, 1873–1884
- Rabinovitz, I., Toker, A., and Mercurio, A. M. (1999) *J. Cell Biol.* **146**, 1147–1160
- Gambaletta, D., Marchetti, A., Benedetti, L., Mercurio, A. M., Sacchi, A., and Falcioni, R. (2000) *J. Biol. Chem.* **275**, 10604–10610
- Falcioni, R., Antonini, A., Nistico, P., Di Stefano, S., Crescenzi, M., Natali, P. G., and Sacchi, A. (1997) *Exp. Cell Res.* **236**, 76–85
- Comoglio P.M., and Boccaccio, C. (2001) *Semin. Cancer Biol.* **11**, 153–165
- Comoglio P.M., and Trusolino, L. (2002) *J. Clin. Invest.* **109**, 857–862
- Flanagan, L., Van Weelden, K., Ammerman, C., Ethier, S. P., and Welsh, J. (1999) *Breast Cancer Res. Treat.* **58**, 193–204
- Lipscomb, E. A., Dugan, A. S., Rabinovitz, I., and Mercurio, A. M. (2003) *Clin. Exp. Metastasis* **20**, 569–576
- Ke, S., Wen, X., Gurfinkel, M., Charnsangavej, C., Wallace, S., Sevik-Muraca, E. M., and Li, C. (2003) *Cancer Res.* **63**, 7870–7875
- O'Connor, K. L., Shaw, L. M., and Mercurio, A. M. (1998) *J. Cell Biol.* **143**, 1749–1760
- Shaw, L. M. (2001) *Mol. Cell Biol.* **21**, 5082–5093
- Chung, J., Bachelder, R. E., Lipscomb, E. A., Shaw, L. M., and Mercurio, A. M. (2002) *J. Cell Biol.* **158**, 165–174
- Jauliac, S., Lopez-Rodriguez, C., Shaw, L. M., Brown, L. F., Rao, A., and Toker, A. (2002) *Nat. Cell Biol.* **4**, 540–544



## Use of RNA interference to inhibit integrin ( $\alpha 6 \beta 4$ )-mediated invasion and migration of breast carcinoma cells

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### Abstract

The application of small interfering RNA (siRNA) oligonucleotides to silence gene expression has profound implications for the intervention of human diseases including cancer. Using this technique, we explored the possibility that the  $\alpha 6 \beta 4$  integrin, a laminin adhesion receptor with a recognized role in the invasive phenotype of many carcinomas, represents a potential therapeutic target to inhibit the migration and invasion of carcinoma cells. We found that siRNA oligonucleotides targeted to either subunit of the  $\alpha 6 \beta 4$  integrin reduced cell surface expression of this integrin and resulted in decreased invasion of MDA-MB-231 breast carcinoma cells. Interestingly, reduced  $\alpha 6 \beta 4$  expression also promoted decreased migration on non-laminin substrata indicating that this integrin can function in a ligand-independent manner. In addition, the absence of  $\beta 4$  expression in these cells augmented the formation of  $\alpha 6 \beta 1$  heterodimers and increased adhesion to laminin-1. Taken together, these results substantiate the importance of the  $\alpha 6 \beta 4$  integrin in invasion and migration that has been demonstrated previously by expression of the  $\beta 4$  subunit in  $\beta 4$ -deficient cell lines and by function blocking antibodies. Furthermore, these data suggest that the utilization of siRNA oligonucleotides to reduce the expression of the  $\alpha 6 \beta 4$  integrin may be a useful approach to prevent carcinoma cell progression.

**Abbreviations:** BSA – bovine serum albumin; DMEM – Dulbecco's modified Eagle's medium; IRS – insulin receptor substrate; LPA – lysophosphatidic acid; PBS – phosphate-buffered saline; PE – phycoerythrin; PI3-K – phosphatidylinositol 3-kinase; RIPA – radioimmune precipitation buffer; RNAi – RNA interference; siRNA – small interfering RNA; si- $\alpha 6$  – siRNA oligonucleotides for  $\alpha 6$ ; si- $\beta 4$  – siRNA oligonucleotides for  $\beta 4$ ; si-Inv – inverted-sequence oligonucleotides for  $\beta 4$ ; si-Scr – scrambled-sequence oligonucleotides for  $\alpha 6$ ; TBS – tris-buffered saline

### Introduction

Double-stranded RNA triggers sequence-specific post-transcriptional gene silencing in a wide variety of organisms [1–3]. This naturally occurring process, referred to as RNA interference (RNAi), has recently been established as a powerful technique for reducing the expression of specific genes in mammalian systems. RNAi is induced in mammalian cells by introducing exogenous 21-nucleotide RNA duplexes or small interfering RNA (siRNA) oligonucleotides that are homologous to the desired gene [4–6]. Because RNAi induction offers great promise as a gene-specific therapeutic tool for a host of disease conditions, we investigated whether gene silencing by siRNA oligonucleotides could be a novel approach to inhibit the migration and invasion of carcinoma cells. As an initial step to addressing this question, we utilized siRNA oligonucleotides for the

$\alpha 6 \beta 4$  integrin, a receptor that has been implicated in the progression of many carcinomas [7].

The  $\alpha 6 \beta 4$  integrin is expressed primarily on the basal surface of most epithelia, and in a few other cell types [7, 8].  $\alpha 6 \beta 4$  is defined as an adhesion receptor for most of the known laminins [9], although increasing evidence indicates that it can also signal independently of ligand binding [10, 11]. The  $\beta 4$  extracellular domain associates exclusively with the  $\alpha 6$  subunit to form  $\alpha 6 \beta 4$  complexes [12] whereas the  $\alpha 6$  subunit also associates with the  $\beta 1$  subunit to form  $\alpha 6 \beta 1$  heterodimers [9]. A primary function of  $\alpha 6 \beta 4$  is to maintain the integrity of epithelia through its ability to mediate the formation of stable and rigid structures termed hemidesmosomes on the basal surface that link the intermediate filament cytoskeleton with laminins in the basement membrane [13].

Although the involvement of  $\alpha 6 \beta 4$  in hemidesmosome organization and function has dominated the study of this integrin, the  $\beta 4$  subunit was initially identified as a tumor-related antigen associated with metastasis [14]. More recently, it has been demonstrated that the expression of  $\alpha 6 \beta 4$  is maintained or even increased in several types of invas-

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ive and metastatic carcinomas and that the  $\alpha 6 \beta 4$  expression level actually correlates with the progression of these carcinomas [7]. These correlative data have been substantiated by functional studies that have defined a pivotal role for  $\alpha 6 \beta 4$  in migration and invasion of carcinoma cells through its ability to interact with F-actin and activate key signaling pathways [15]. Furthermore,  $\alpha 6 \beta 4$  promotes survival in carcinoma cells lacking functional p53 protein [16]. The importance of  $\alpha 6 \beta 4$  in survival has recently been extended by the report that  $\alpha 6 \beta 4$  stimulates translation of vascular endothelial growth factor and that this pathway is a mechanism of survival in carcinoma cells [17].

The role of  $\alpha 6 \beta 4$  in migration and invasion was initially shown by exogenous expression of this integrin in  $\beta 4$ -deficient colon and breast carcinoma cells [18, 19]. In breast carcinoma studies,  $\alpha 6 \beta 4$  promoted invasion in a phosphatidylinositol 3-kinase (PI3-K)-dependent manner [19]. Since these initial studies,  $\alpha 6 \beta 4$  has been shown to activate PI3-K and stimulate migration and invasion by both growth factor receptor-dependent and -independent signaling mechanisms. For instance,  $\alpha 6 \beta 4$  and ErbB-2, an orphan receptor of the epidermal growth factor family, associate in carcinoma cells [20] and this interaction stimulates PI3-K activity and invasion in a fibroblast model system [21]. An association of  $\alpha 6 \beta 4$  with the Met tyrosine kinase in carcinoma cells has also been demonstrated and  $\alpha 6 \beta 4$  was reported to be necessary for the invasive functions of Met by acting as an adapter protein to recruit PI3-K for enhanced Met signaling [11]. In a growth factor receptor-independent manner, the insulin receptor substrates (IRS-1 and IRS-2) act as signaling intermediates to link activated  $\alpha 6 \beta 4$  with PI3-K, a pathway that leads to an increase in carcinoma invasion [22]. In addition to stimulation of PI3-K signaling, the  $\alpha 6 \beta 4$  integrin has also been shown to promote migration by activation of the MAPK pathway [23, 24], the Rac and RhoA GTPases [19, 25], and the nuclear factor of activated T-cells family of transcription factors [26].

Although the data summarized above indicate that  $\alpha 6 \beta 4$  plays a pivotal function in the aggressive behavior of carcinoma cells, it is worth noting that this role was deduced from either expression of the  $\beta 4$  subunit into  $\beta 4$ -negative cells or the use of antibodies to inhibit  $\alpha 6 \beta 4$  function. In the current study, we used the more definitive approach of RNAi to target the  $\alpha 6$  and  $\beta 4$  subunits of the  $\alpha 6 \beta 4$  integrin in breast carcinoma cells. Using this highly specific and efficient approach, we observed a significant reduction in the surface expression of this integrin that correlated with an inhibition of migration and invasion. Interestingly, the impact of reduced  $\alpha 6 \beta 4$  expression was apparent on non-laminin substrates, a finding that substantiates the ligand-independent function of  $\alpha 6 \beta 4$ . These findings indicate that inhibition of integrin expression by siRNA oligonucleotides may be an effective approach to assess integrin function in carcinoma cells, as well as to impede tumor progression *in vivo*.

## Materials and methods

### Cells

The MDA-MB-231 human breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository at Georgetown University and maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Rockville, Maryland) supplemented with 10% fetal bovine serum (Sigma, St. Louis, Missouri), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (each from Life Technologies).

### siRNA transfections

Oligonucleotide sequences for  $\beta 4$  integrin were designated as si- $\beta 4$  (GAGCUGCACGGAGUGUGUC) and as si-Inv (CUGUGUGAGGCACGUCGAG), an inverted control.  $\alpha 6$  integrin oligonucleotides included si- $\alpha 6$  (GGUCGUGACAUGUGCUCAC) and a scrambled-sequence control, si-Scr (AUGCAGAGUGGCGCUCUCU). Oligonucleotides were synthesized by Dharmacon Research, Inc. (Lafayette, Colorado). Cells ( $0.5\text{--}2.0 \times 10^5$ ) were plated onto 35-mm tissue culture dishes 24 h prior to transfection with 200 nM of siRNA duplex using 25  $\mu$ g of TransIT-TKO transfection reagent (Mirus, Madison, Wisconsin) in the presence of serum as described by the manufacturer. One day following transfection, the transfection medium was aspirated from the cells and replaced with fresh complete growth medium and incubated for an additional 48–72 h. For each transfection, either immunoblotting or flow cytometry was used to confirm reduced protein expression of the targeted gene.

### Cell Surface Biotinylation and Immunoprecipitation

Following transfection for 3–4 days, MDA-MB-231 cells were washed two times each with phosphate-buffered saline (PBS) and HEPES buffer (20 mM HEPES, 130 mM NaCl, 5 mM KCl, 0.8 mM Mg Cl<sub>2</sub>, 1.0 mM CaCl<sub>2</sub>, pH 7.45). The cells were then incubated on ice with HEPES buffer containing EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, Illinois) at 0.5 mg/ml for 30 min. Each dish was washed three times with HEPES buffer and the cells were lysed in ice-cold radioimmune precipitation (RIPA) buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% Nonidet-P40, 1% deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 5  $\mu$ g/ml aprotinin, leupeptin, and pepstatin) for 15 min at 4°C. Cell lysates were clarified by centrifugation at  $10,000 \times g$  for 10 min, the supernatants collected, and the total protein concentration of each lysate determined by the Bio-Rad DC protein assay (Hercules, California).

Immunoprecipitations following cell surface labeling were performed with equal amounts of total protein or 60  $\mu$ g for  $\alpha 3$  and  $\beta 1$  integrin reactions and 125–150  $\mu$ g for  $\alpha 6$  and  $\beta 4$  integrin samples. Lysates were preabsorbed with either rat IgG whole molecule agarose (Sigma) or, for mouse antibodies, protein G-Sepharose (Amersham, Piscataway, New

Jersey) and then incubated with 1  $\mu$ g of an anti-integrin antibody overnight at 4°C. The following antibodies were used for immunoprecipitation: 439-9B, rat anti- $\beta$ 4 integrin mAb (obtained from Rita Falcioni, Regina Elena Cancer Institute, Rome, Italy); GoH3, rat anti- $\alpha$ 6 integrin mAb (Immunotech, Westbrook, Maine); MC13, mouse anti- $\beta$ 1 integrin mAb (obtained from Steve Akiyama, NIH, Research Triangle Park, North Carolina); P1B5, mouse anti- $\alpha$ 3 integrin mAb (Life Technologies); as well as rat and mouse IgG (Sigma). Immune complexes were precipitated with rat IgG agarose or protein G-Sepharose, washed four times with RIPA buffer, and eluted in 1X reducing sample buffer (biotinylated  $\alpha$ 6 and  $\beta$ 4 integrin immunoprecipitations) or 1X non-reducing sample buffer (biotinylated  $\alpha$ 3 and  $\beta$ 1 integrin immunoprecipitations).

#### Immunoblotting

For preparing whole cell lysates, cells were rinsed twice with PBS and lysed in RIPA buffer as described above. Lysates and immune complexes were separated by SDS-PAGE and transferred to Hybond-C nitrocellulose membranes (Amersham). Membranes were incubated in block buffer consisting of Tris-buffered saline (TBS) containing 5% nonfat dry milk followed by antibody incubation in TBS containing 1% nonfat dry milk and 0.05% Tween-20. The blots were incubated with a 1:5000 dilution of rabbit polyclonal anti- $\beta$ 4-integrin (505) [15] or 0.5  $\mu$ g/ml anti- $\beta$ -actin (Sigma) followed by 0.04  $\mu$ g/ml peroxidase-conjugated donkey anti-rabbit secondary antibody (Jackson Immnoresearch, West Grove, Pennsylvania). For biotinylation studies, the membranes were incubated in block buffer overnight and then labeled with 0.2  $\mu$ g/ml peroxidase-conjugated streptavidin in block buffer containing 0.05% Tween-20 for 2 hours at 25°C. All membranes were visualized by chemiluminescence (SuperSignal West Pico, Pierce). Densitometry was performed using IP Lab Spectrum (Webster, New York) computer software.

#### Apoptosis assay

Four days following transfection, cells were collected and their level of apoptosis was assessed using AnnexinV-phycoerythrin (PE) (Pharmingen, San Diego, California). Briefly, cells were washed one time each in 1X PBS and IX annexin buffer (10 mM Hepes-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ) and then incubated for 15 minutes at room temperature with 5  $\mu$ g/ml AnnexinV-PE. Following incubation, cells were washed once with 1X annexin buffer and analyzed by flow cytometry.

#### Migration and invasion assays

For chemotaxis assays, the upper and lower surface of the membrane in each Transwell chamber (Costar, Cambridge, Massachusetts) were coated overnight at 4°C with 15  $\mu$ g/ml of collagen I (Vitrogen, Palo Alto, California). To prepare the Transwell membranes for invasion assays, 0.5  $\mu$ g of Matrigel (Collaborative Research, Bedford, Massachusetts) was diluted with cold water and dried onto each filter

overnight at 25°C. For both chemotaxis and invasion assays, Transwell membranes were blocked on the following day with DMEM for 1 hour at 37°C. Cells were trypsinized and resuspended in DMEM containing 0.25% heat-inactivated lipid free bovine serum albumin and a total of  $1 \times 10^5$  cells was added to the upper chamber of each well. NIH3T3 conditioned medium or 100 nM lysophosphatidic acid (LPA) (Sigma) was added to the lower chamber of each well. After incubating for 1 hour (chemotaxis assays) or 4 hours (invasion assays) at 37°C, nonmigrating cells were removed from the upper chamber with a cotton swab. Cells that had migrated to the lower surface of the membrane were fixed with 100% methanol and stained with 0.2% crystal violet in 2% ethanol. Migration was quantified by counting cells per square millimeter using bright-field optics.

#### Adhesion assays

For adhesion assays, 96-well tissue culture plates were coated overnight at 4°C with 20  $\mu$ g/ml laminin-1 (prepared from the EHS sarcoma provided by Dr Hynda Kleinman, NIDR, Bethesda, Maryland) or 1% BSA as a negative control. The wells were then washed 3 times with PBS and blocked for 10 min at 37°C with 250  $\mu$ g/ml heat-inactivated lipid free BSA in DMEM. Cells ( $3 \times 10^4$ ) were resuspended in DMEM and added to the protein-coated wells. After a 1 hour incubation at 37°C, the wells were washed 3 times with PBS, fixed for 10 min with methanol, stained with a solution of 0.2% crystal violet in 2% ethanol, and washed 3 times with water. The crystal violet stain was solubilized with a 1% SDS solution and the adhesion was quantified by measuring the absorbance at 595 nm. To control for nonspecific adhesion, the mean absorbance (four wells per transfection condition) of the BSA-coated wells was subtracted from the mean absorbance of the laminin-coated wells in each experiment. The BSA-corrected adhesion obtained in the cells transfected with sequence-inverted  $\beta$ 4 siRNA oligonucleotides was designated as equal to one and used to determine the fold induction of untransfected and  $\beta$ 4 siRNA oligonucleotide transfected cells.

## Results

#### siRNA oligonucleotides for $\beta$ 4 (si- $\beta$ 4) decrease $\alpha$ 6 $\beta$ 4 cell surface expression

We used MDA-MB-231 cells to assess the potential usefulness of RNAi as an approach to inhibit  $\alpha$ 6 $\beta$ 4 function in carcinoma cells. These invasive and metastatic breast carcinoma cells express relatively high levels of  $\alpha$ 6 $\beta$ 4, as well as a small population of  $\alpha$ 6 $\beta$ 1 (L. Shaw, personal communication). The total level of endogenous  $\beta$ 4 subunit was reduced by approximately 75% 4 days following transfection with si- $\beta$ 4 oligonucleotides compared to control inverted-sequence oligonucleotides (si-Inv) or untransfected (Unt) cells (Figure 1A). Reduced  $\beta$ 4 expression was not detected earlier than 96 h (data not shown) indicating that siRNA oligonucleotides directed at  $\beta$ 4, and possibly other membrane

spanning receptors, require several days to suppress protein expression effectively. These data demonstrate that si- $\beta$ 4 oligonucleotides reduce endogenous  $\beta$ 4 expression in a breast carcinoma cell line.

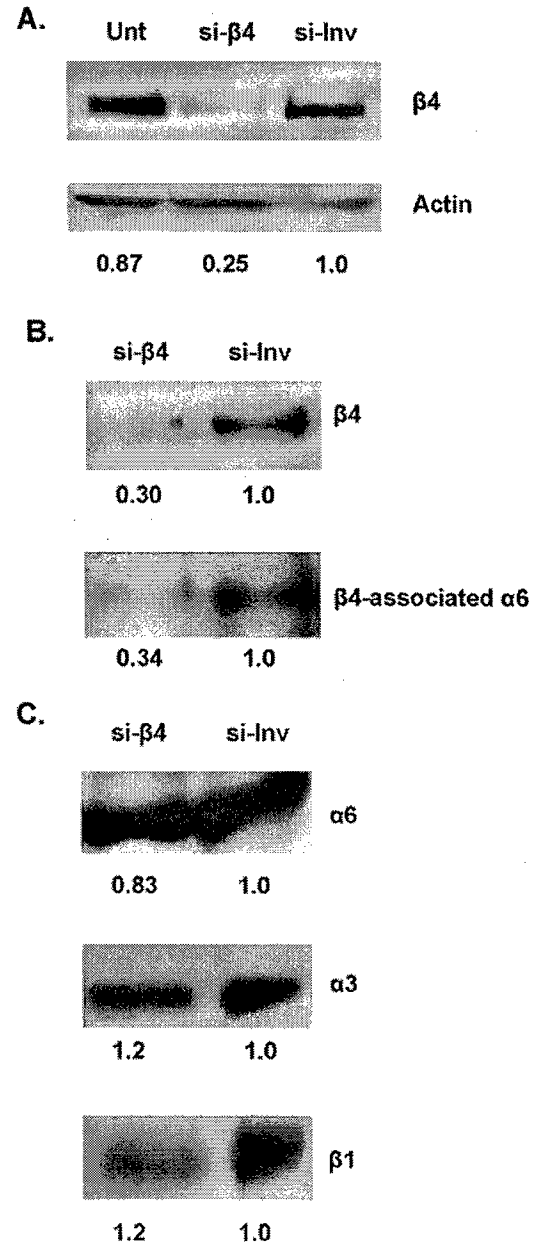
To determine whether expression of the  $\alpha$ 6 $\beta$ 4 integrin was suppressed on the cell surface of MDA-MB-231 cells following transfection with si- $\beta$ 4 oligonucleotides, cells were biotinylated and immunoprecipitated for  $\beta$ 4.  $\beta$ 4 expression was decreased on the cell surface of si- $\beta$ 4 transfected cells by 70% compared to si-Inv cells and the amount of  $\alpha$ 6 that co-immunoprecipitated with  $\beta$ 4 in these cells was decreased by 66% (Figure 1B). These findings indicate that cell surface expression of  $\alpha$ 6 $\beta$ 4 is diminished in si- $\beta$ 4 transfected breast carcinoma cells.

We next investigated whether the surface expression of the  $\alpha$ 6 subunit was altered in si- $\beta$ 4 transfected cells. Immunoprecipitation of biotin-labeled lysates revealed no significant difference in  $\alpha$ 6 expression between si- $\beta$ 4 and si-Inv transfected cells (Figure 1C). Moreover, the surface expression of the  $\alpha$ 3 and  $\beta$ 1 integrin subunits was maintained in cells transfected with si- $\beta$ 4 oligonucleotides (Figure 1C). Isotype-control immunoprecipitations revealed the specificity of the immunoprecipitated protein for each integrin antibody (data not shown). Additional experiments using flow cytometry to identify cell surface integrin expression confirmed these results (data not shown). These data demonstrate that si- $\beta$ 4 oligonucleotides reduce the expression of  $\alpha$ 6 $\beta$ 4 on the cell surface of breast carcinoma cells.

*si- $\beta$ 4 oligonucleotides decrease invasion and ligand-independent migration of MDA-MB-231 breast carcinoma cells*

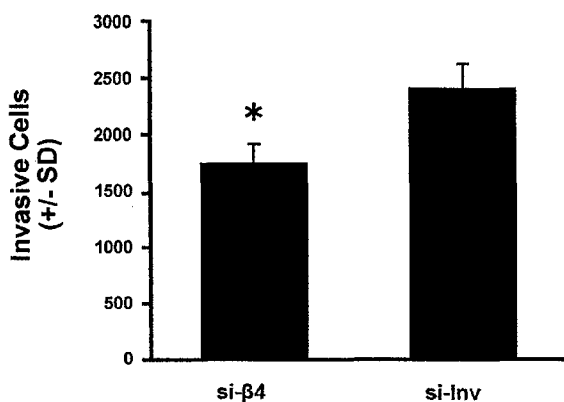
To address the functional consequence of reduced  $\alpha$ 6 $\beta$ 4 expression, we evaluated the ability of MDA-MB-231 cells to invade through Matrigel-coated Transwells following transfection with si- $\beta$ 4 oligonucleotides. The invasion of si- $\beta$ 4 transfected cells towards fibroblast conditioned medium was inhibited by 27% compared to cells transfected with the inverted control (Figure 2A). Based on reports that the  $\alpha$ 6 $\beta$ 4 integrin can impact the survival of carcinoma cells in stress conditions [16, 17], it was important to evaluate the effect of these oligonucleotides on apoptosis. The level of apoptosis was 5% for both si- $\beta$ 4 and si-Inv transfected cells as determined by the percentage of AnnexinV-PE<sup>+</sup> cells (data not shown). This data indicate that reduction of  $\alpha$ 6 $\beta$ 4 expression does not result in increased apoptosis under these conditions (10% serum) and that the decrease in invasion of si- $\beta$ 4 transfected cells cannot be attributed to increased levels of cell death.

The ability of  $\alpha$ 6 $\beta$ 4 to influence the functions of carcinoma cells can occur independently of ligand (laminin) binding [10, 11]. In particular,  $\alpha$ 6 $\beta$ 4 stimulates the chemotactic migration of carcinoma cells on collagen I but appears to have little impact on their haptotactic migration towards collagen, a process that involves only collagen adhesion receptors [10]. For this reason, we assessed the ability of MDA-MB-231 cells transfected with si- $\beta$ 4 and

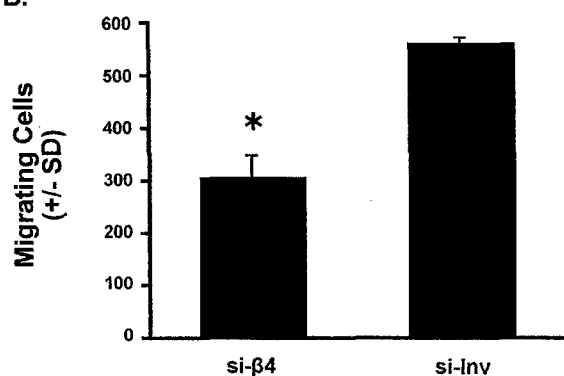


**Figure 1.**  $\alpha$ 6 $\beta$ 4 expression is reduced by si- $\beta$ 4 oligonucleotides. (A) MDA-MB-231 breast carcinoma cells were either untransfected (Unt) or transfected with  $\beta$ 4 siRNA oligonucleotides (si- $\beta$ 4) or sequence-inverted siRNA oligonucleotides (si-Inv). RIPA extracts were obtained 96 hours post-transfection and equal amounts (30  $\mu$ g) of total protein were resolved under reducing conditions by 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for  $\beta$ 4 and  $\beta$ -actin. The fold change in  $\beta$ 4 expression was determined by normalizing each band to  $\beta$ -actin and designating the si-Inv lane as equal to 1. Similar results (25–75% reduction in  $\beta$ 4 expression) were observed in greater than 5 independent experiments. Four days following transfection with si- $\beta$ 4 or si-Inv oligonucleotides, MDA-MB-231 cells were biotinylated, extracted in RIPA buffer, and immunoprecipitated for  $\beta$ 4 (B) or  $\alpha$ 6,  $\alpha$ 3, and  $\beta$ 1 (C), and transferred to nitrocellulose. Immunoprecipitations for  $\alpha$ 6 and  $\beta$ 4 (150  $\mu$ g of total protein) were separated by 6% SDS-PAGE under reducing conditions whereas  $\alpha$ 3 and  $\beta$ 1 immunoprecipitations (60  $\mu$ g of total protein) were eluted in non-reducing sample buffer. The membranes were incubated with peroxidase-conjugated streptavidin. The fold change in integrin expression following si- $\beta$ 4 transfection, when the si-Inv lane is set equal to 1, is indicated. Shown are representative blots from 3 separate experiments.

A.



B.



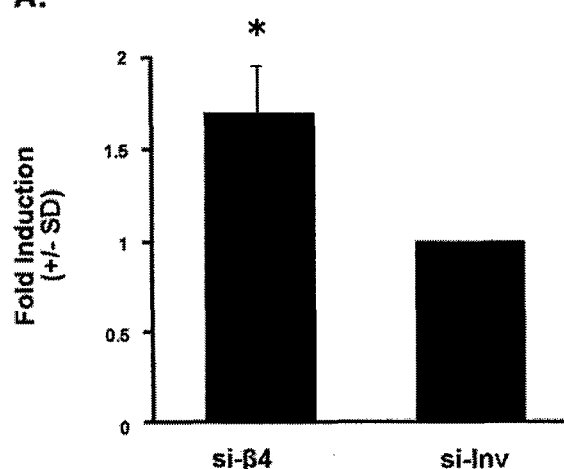
**Figure 2.** si-β4 oligonucleotides decrease invasion and migration in MDA-MB-231 cells. (A) Four days following transfection, the ability of si-β4 and si-Inv transfected MDA-MB-231 cells to invade Matrigel towards conditioned fibroblast medium was investigated in a 4 h assay. The results represent the mean number of invasive cells (+/- SD) from two wells (five fields per well). The number of invasive cells was significantly less for si-β4 transfected cells than for si-Inv transfected cells (\*two-tailed *t*-test, *P* = 0.03). Similar data were obtained in four separate experiments. (B) At 96 hours post-transfection, MDA-MB-231 cells were allowed to migrate through collagen I-coated Transwell membranes for 1 hour towards LPA (100 nM). The data represent the mean number of migrating cells (+/- SD) from 2 wells (5 fields per well). The number of migrating cells was significantly less for si-β4 transfected cells than for si-Inv transfected cells (\*two-tailed *t*-test, *P* = 0.004). Similar results were obtained in three independent trials.

si-Inv oligonucleotides to migrate towards LPA, a known chemoattractant of breast carcinoma cells [10], on collagen I. The ligand-independent migration of MDA-MB-231 cells was inhibited by 45% when transfected with si-β4 oligonucleotides compared to si-Inv oligonucleotides (Figure 2B). Taken together, these observations confirm the importance of α6β4 expression in the migration and invasion of carcinoma cells as demonstrated previously by antibody inhibition experiments and by exogenous expression of this integrin in β4-deficient carcinoma cell lines [18, 19].

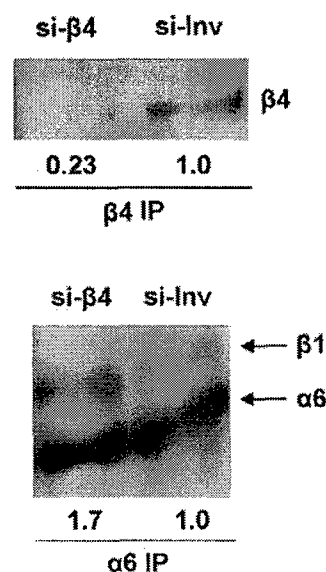
*Loss of β4 expression leads to increased adhesion to laminin-1 in MDA-MB-231 breast carcinoma cells*

Because α6β4 can function as a receptor for laminin-1 in some cells [9], we hypothesized that reduced α6β4 expres-

A.



B.



**Figure 3.** Reduced β4 expression promotes adhesion to laminin-1 by enhancing α6β1 formation. (A) MDA-MB-231 cells transfected with si-β4 or si-Inv oligonucleotides were incubated for 1 hour in laminin-1 coated wells. Data are the mean fold induction (+/- SD) from three independent experiments, corrected for nonspecific adhesion to BSA, where the adhesion of si-Inv transfected cells was designated as equal to 1. The fold induction of si-β4 transfected cells was significantly greater than observed for si-Inv transfected cells (\*, two-tailed *t*-test, *P* = 0.04). (B) si-β4 and si-Inv transfected MDA-MB-231 cells were biotinylated and lysed 96 h post-transfection and 150 μg of total protein was immunoprecipitated for either the β4 (top blot) or α6 (bottom blot) subunit. Immune complexes were separated by 6% SDS-PAGE under reducing conditions and transferred to nitrocellulose. The membranes were then incubated with peroxidase-conjugated streptavidin and visualized by chemiluminescence. The level of β4 (top) and α6-associated β1 (bottom) expression following immunoprecipitation with the indicated antibodies is shown below each lane where the amount of β4 (top) and β1 (bottom) in the si-Inv sample is set equal to 1.

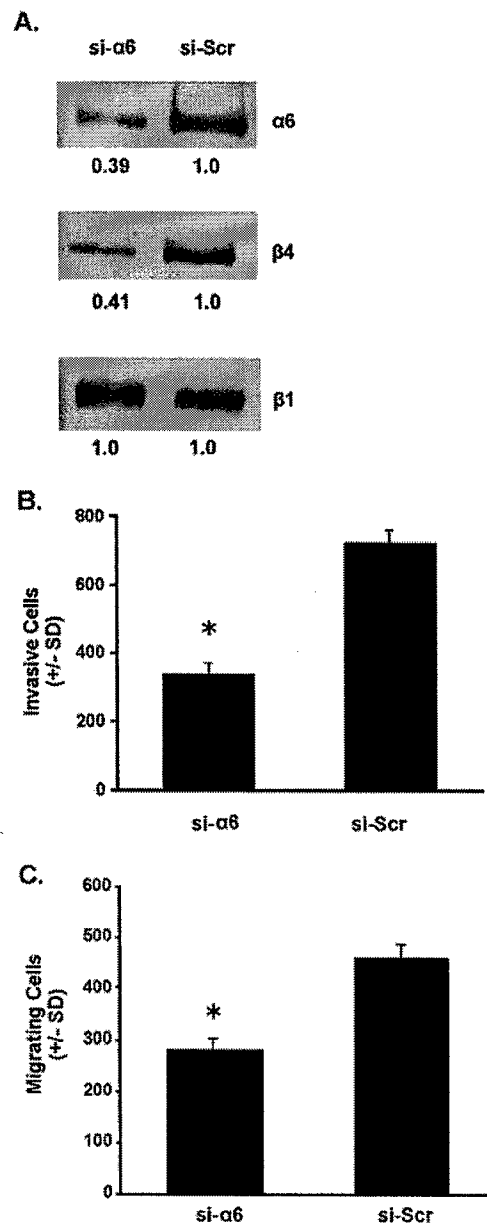


sion by siRNA oligonucleotide transfection would disrupt adhesion to this laminin. However, the level of adhesion to laminin-1 in si- $\beta 4$  transfected cells was significantly higher (1.7 fold) than observed in si-Inv transfected cells (Figure 3A). Considering that the expression of the  $\alpha 6$  subunit remains unchanged in si- $\beta 4$  transfected cells (Figure 1C and Figure 3B), we reasoned that the reduction of  $\alpha 6\beta 4$  might lead to redistribution of the  $\alpha 6$  subunit towards  $\alpha 6\beta 1$ , also a laminin-1 receptor [9]. We found that cells with decreased  $\beta 4$  expression (Figure 3B) had almost twice the level of  $\alpha 6$ -associated  $\beta 1$  as compared to si-Inv transfected cells as shown by immunoprecipitation of the  $\alpha 6$  subunit in the same biotinylated lysates (Figure 3B). Co-immunoprecipitation of the  $\alpha 6$  subunit in si- $\beta 4$  and si-Inv transfected cells followed by immunoblotting for the  $\beta 1$  subunit yielded similar results (data not shown). These findings indicate that in the absence of sufficient levels of  $\beta 4$ ,  $\alpha 6$  forms additional heterodimers with  $\beta 1$  in breast carcinoma cells. These findings indicate that in the absence of sufficient levels of  $\beta 4$ ,  $\alpha 6$  forms additional heterodimers with  $\beta 1$  in breast carcinoma cells.

*$\alpha 6$  siRNA oligonucleotides (si- $\alpha 6$ ) inhibit invasion and migration by reducing  $\alpha 6\beta 4$  cell surface expression*

MDA-MB-231 cells were transfected with si- $\alpha 6$  oligonucleotides and  $\alpha 6$  scrambled-sequence oligonucleotides (si-Scr) to determine whether reduced  $\alpha 6$  expression alters  $\beta 4$  cell surface levels and consequently decreases invasion and migration. Three days following transfection, cell surface receptors were biotinylated and cell lysates were immunoprecipitated for  $\alpha 6$ ,  $\beta 4$ , and  $\beta 1$ . The si- $\alpha 6$  oligonucleotides decreased the expression of  $\alpha 6$  by 61% compared to cells transfected with control oligonucleotides (Figure 4A). Interestingly,  $\beta 4$  expression was decreased by 59% in si- $\alpha 6$  transfected cells compared to si-Scr transfected cells whereas the cell surface expression of  $\beta 1$  was unchanged in cells transfected with either si- $\alpha 6$  or si-Scr oligonucleotides. Isotype-control immunoprecipitations performed for each antibody were negative (data not shown). These data indicate that siRNA oligonucleotides for  $\alpha 6$  effectively reduce the level of  $\alpha 6\beta 4$  expression on the cell surface of breast carcinoma cells. In addition, these findings substantiate the fact that most, if not all,  $\beta 4$  expressed on the cell surface is associated with  $\alpha 6$ .

Given our finding that si- $\alpha 6$  oligonucleotides efficiently decrease the level of  $\alpha 6\beta 4$  on the cell surface of MDA-MB-231 cells, we investigated the ability of si- $\alpha 6$  oligonucleotides to inhibit their invasion and migration. The invasion of si- $\alpha 6$  transfected cells through Matrigel towards LPA was decreased by 53% compared to cells transfected with si-Scr oligonucleotides (Figure 4B). In additional experiments, we found a similar reduction in invasion when fibroblast conditioned medium was used as the chemoattractant (data not shown). The level of apoptosis was comparable for both si- $\alpha 6$  and si-Scr transfected cells as determined by the percentage of AnnexinV-PE<sup>+</sup> cells (data not shown). To assess the ability of si- $\alpha 6$  and si-Scr transfected cells to migrate in a ligand-independent manner, we performed migration assays towards LPA on collagen I-coated Transwells. The mean



**Figure 4.** si- $\alpha 6$  oligonucleotides decrease invasion and migration of MDA-MB-231 cells. (A) Three days following transfection with si- $\alpha 6$  or si-Scr oligonucleotides, MDA-MB-231 cells were biotinylated, extracted in RIPA buffer, and immunoprecipitated for  $\alpha 6$ ,  $\beta 4$ , and  $\beta 1$ . Each immunoprecipitation was initiated with 125  $\mu$ g of total protein and separated by 6% SDS-PAGE. The membranes were incubated with peroxidase-conjugated streptavidin. The fold change in integrin expression following si- $\alpha 6$  transfection is shown in comparison to the si-Scr transfected band. (B) The ability of si- $\alpha 6$  and si-Scr transfected MDA-MB-231 cells to invade Matrigel towards LPA in a 4 h assay was assessed. The results represent the mean number of invasive cells (+/- SD) from two wells (five fields per well). The mean number of invasive cells was significantly decreased for si- $\alpha 6$  transfected cells compared to si-Scr transfected cells (\*two-tailed *t*-test,  $P = 0.0002$ ). Similar results were obtained in three independent trials. (C) At 72 h post-transfection with si- $\alpha 6$  or si-Scr oligonucleotides, the migration of these cells through collagen I-coated Transwell membranes towards LPA (100 nM) for 1 h was determined. The data represent the mean number of migrating cells (+/- SD) from two wells (five fields per well). The number of migrating cells was significantly less for si- $\alpha 6$  transfected cells than for si-Scr transfected cells (\*two-tailed *t*-test,  $P = 0.001$ ). Similar results were obtained in two separate experiments.

number of migrating cells was decreased by 39% in the si- $\alpha 6$  transfected cells compared to the si-Scr transfected cells (Figure 4C). These results provide more definitive evidence for the ability of  $\alpha 6\beta 4$  to stimulate migration on non-laminin substrata. Overall, these data emphasize the importance of  $\alpha 6\beta 4$  in the invasive phenotype of breast carcinoma cells.

## Discussion

The relatively new technique of RNAi is a potentially powerful tool to assess the contribution of specific molecules to invasion and metastasis. In particular, the specificity and efficacy of this approach may be especially valuable for studying the integrin family members that exhibit complex structures and multiple functions. To assess the feasibility of RNAi in this capacity, we focused on the  $\alpha 6\beta 4$  integrin. The involvement of this integrin in invasion and migration has been previously established by expression of the  $\beta 4$  subunit in  $\beta 4$ -deficient cell lines and by function blocking antibodies. In this study, we provide the first evidence that the integrins can be inhibited by the more definite approach of RNAi and suggest that targeting of the  $\alpha 6\beta 4$  integrin may be an effective strategy to assess the functions of this integrin and impede carcinoma progression.

The utilization of siRNA oligonucleotides targeted to the  $\alpha 6$  and  $\beta 4$  subunits of the  $\alpha 6\beta 4$  integrin provided valuable information not only for the usefulness of RNAi to inhibit surface receptors but also on the mechanistic function of this integrin. We found that the endogenous expression of  $\alpha 6\beta 4$  was not decreased until 72 or 96 hours post-transfection with si- $\alpha 6$  and si- $\beta 4$  oligonucleotides, respectively. This observation indicates that surface receptors may require considerable time for the RNAi approach to be effective and suggests that the half-life of the target protein and the rate of surface receptor recycling are major determinants in the ability of RNAi to inhibit expression. In contrast, our laboratory has shown that soluble proteins are inhibited efficiently by RNAi, usually within 24–48 hours after transfection (R.E. Bachelder, unpublished observation). In functional assays, reduced  $\alpha 6\beta 4$  expression resulted in significant decreases in invasion and migration even though the inhibition of  $\alpha 6\beta 4$  expression was less than complete. If the expression of  $\alpha 6\beta 4$  were further diminished, the effects would likely be more dramatic. Because the reduction in  $\alpha 6\beta 4$  expression in these studies is dependent on both transfection efficiency and the specific gene sequence selected for RNAi inhibition, manipulation of either of these parameters may ultimately lead to greater reduction in  $\alpha 6\beta 4$  expression. For example, the use of different siRNA oligonucleotides targeted to either of the  $\alpha 6\beta 4$  subunits may provide more complete inhibition. In addition, the recently developed DNA vector-based RNAi technology [27–29] which allows for stable inhibition of the targeted gene will eliminate transfection efficiency concerns and should also permit the generation of  $\alpha 6\beta 4$  deficient cell lines. The implementation of these strategies will likely enable us to achieve greater reduction in  $\alpha 6\beta 4$  expression in future studies.

Data from our laboratory and others have recently implicated the  $\beta 4$ -subunit in ligand-independent signaling. For example, the  $\alpha 6\beta 4$  integrin promotes migration and invasion, as well as lamellae formation, on non-laminin substrata such as collagen and these functions cannot be blocked by antibodies that inhibit  $\alpha 6\beta 4$  adhesive interactions, an observation that discounts the possibility of adhesion to laminins deposited by the cells [10]. More recently, it was demonstrated that a truncated form of  $\beta 4$  that was unable to bind laminins could promote invasion [11]. In this study, the migration of MDA-MB-231 cells on collagen I, a non-ligand for  $\alpha 6\beta 4$ , was significantly inhibited by si- $\beta 4$  and si- $\alpha 6$  oligonucleotides thus providing further evidence that this integrin can function in the absence of ligand. This concept has profound implications for migration and invasion because it implies that the ability of  $\alpha 6\beta 4$  to stimulate these pathways is not limited to specific matrix environments. Although the mechanism by which  $\alpha 6\beta 4$  functions in this manner is unknown, the observation that  $\beta 4$  cytoplasmic domains can self-associate may explain this ligand-independent signaling [30]. Furthermore,  $\alpha 6\beta 4$  has been shown to regulate the function of  $\alpha 3\beta 1$ , a dual collagen/laminin receptor, in keratinocytes [31, 32]. Even though we did not observe a change in either  $\alpha 3$  or  $\beta 1$  subunit expression in our studies, we cannot rule out the possibility that  $\alpha 6\beta 4$  affects  $\alpha 3\beta 1$ -mediated signaling to cause decreased migration on collagen I in si- $\beta 4$  and si- $\alpha 6$  transfected cells.

Because  $\alpha 6\beta 4$  is an adhesion receptor for laminin-1 as well as other laminins [9], we hypothesized that loss of this integrin would promote a decrease in adhesion to this substrata. However, we observed an increase in adhesion to laminin-1 following transfection with si- $\beta 4$  oligonucleotides. This seeming paradox is explained by our finding that the  $\alpha 6$  subunit forms additional  $\alpha 6\beta 1$  complexes in the absence of the  $\beta 4$  subunit. This finding is further supported by the observation that the  $\alpha 6\beta 4$  integrin is decreased with either si- $\beta 4$  or si- $\alpha 6$  oligonucleotides but that cells transfected with si- $\alpha 6$  oligonucleotides have reduced levels of the  $\beta 4$  subunit whereas the si- $\beta 4$  oligonucleotides do not significantly decrease the expression of the  $\alpha 6$  subunit. Thus, the  $\alpha 6$  subunit is stabilized on the cell surface by associating with  $\beta 1$  subunits that are found expressed in excess in many cells but the  $\beta 4$  subunit, that associates exclusively with the  $\alpha 6$  subunit [12], is lost from the cell surface when the  $\alpha 6$  subunit is removed.

Several groups have recently demonstrated that siRNA oligonucleotides can inhibit gene expression *in vivo*. For example, mouse tail vein injections with siRNA oligonucleotides for luciferase, delivered as naked siRNA oligonucleotides or as plasmid DNA, in combination with a luciferase expression vector significantly reduced luciferase expression in adult mice [33]. In similar studies, the endogenous expression of the green fluorescent protein was decreased in the liver of a transgenic mouse strain that expresses this gene in nearly all organs [34]. Furthermore, the therapeutic potential of this technique to inhibit cancer progression was specifically shown by the lack of tumor formation in nude mice that were injected with cells that had been selected for stable

and reduced expression of oncogenic K-RAS [35]. Taken together, these studies indicate that RNAi may be a viable approach to treat human diseases including cancer.

In summary, we have shown that siRNA oligonucleotides can reduce the endogenous surface expression of the  $\alpha 6 \beta 4$  integrin in carcinoma cells. In the absence of  $\alpha 6 \beta 4$ , we observed a significant reduction in the invasion and ligand-independent migration of these cells. These results demonstrate the validity of this technique to inhibit integrin expression in carcinoma cells and suggest that the  $\alpha 6 \beta 4$  integrin may be a potential gene target to prevent tumor progression *in vivo*. Future studies by our laboratory will directly explore this possibility in mouse cancer models.

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### References

- Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet* 2001; 2: 110–9.
- Hutvagner G, Zamore PD. RNAi: Nature abhors a double-strand. *Curr Opin Genet Dev* 2002; 12: 225–32.
- Sharp PA. RNA interference-2001. *Genes Dev* 2001; 15: 485–90.
- Caplen NJ, Parrish S, Imani F et al. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci USA* 2001; 98: 9742–7.
- Elbashir SM, Harborth J, Lendeckel W et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; 411: 494–8.
- Tuschl T, Zamore PD, Lehmann R et al. Targeted mRNA degradation by double-stranded RNA *in vitro*. *Genes Dev* 1999; 13: 3191–7.
- Mercurio AM, Rabinovitz I. Towards a mechanistic understanding of tumor invasion-lessons from the  $\alpha 6 \beta 4$  integrin. *Semin Cancer Biol* 2001; 11: 129–41.
- Mercurio AM, Rabinovitz I, Shaw LM. The  $\alpha 6 \beta 4$  integrin and epithelial cell migration. *Curr Opin Cell Biol* 2001; 13: 541–5.
- Mercurio A. Receptors for the laminins. Achieving specificity through cooperation. *Trends Cell Biol* 1995; 5: 419–23.
- O'Connor KL, Shaw LM, Mercurio AM. Release of cAMP gating by the  $\alpha 6 \beta 4$  integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *J Cell Biol* 1998; 143: 1749–60.
- Trusolino L, Bertotti A, Comoglio PM. A signaling adapter function for  $\alpha 6 \beta 4$  integrin in the control of HGF-dependent invasive growth. *Cell* 2001; 107: 643–54.
- Hemler ME, Crouse C, Sonnenberg A. Association of the VLA  $\alpha 6$  subunit with a novel protein. A possible alternative to the common VLA  $\beta 1$  subunit on certain cell lines. *J Biol Chem* 1989; 264: 6529–35.
- Borradori L, Sonnenberg A. Structure and function of hemidesmosomes: More than simple adhesion complexes. *J Invest Dermatol* 1999; 112: 411–8.
- Falcioni R, Sacchi A, Resau J, Kennel SJ. Monoclonal antibody to human carcinoma-associated protein complex: Quantitation in normal and tumor tissue. *Cancer Res* 1988; 48: 816–21.
- Rabinovitz I, Toker A, Mercurio AM. Protein kinase C-dependent mobilization of the  $\alpha 6 \beta 4$  integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. *J Cell Biol* 1999; 146: 1147–60.
- Bachelder RE, Ribick MJ, Marchetti A et al. p53 inhibits  $\alpha 6 \beta 4$  integrin survival signaling by promoting the caspase 3-dependent cleavage of AKT/PKB. *J Cell Biol* 1999; 147: 1063–72.
- Chung J, Bachelder RE, Lipscomb EA et al. Integrin ( $\alpha 6 \beta 4$ ) regulation of eIF-4E activity and VEGF translation: A survival mechanism for carcinoma cells. *J Cell Biol* 2002; 158: 165–74.
- Chao C, Lotz MM, Clarke AC, Mercurio AM. A function for the integrin  $\alpha 6 \beta 4$  in the invasive properties of colorectal carcinoma cells. *Cancer Res* 1996; 56: 4811–9.
- Shaw LM, Rabinovitz I, Wang HH et al. Activation of phosphoinositide 3-OH kinase by the  $\alpha 6 \beta 4$  integrin promotes carcinoma invasion. *Cell* 1997; 91: 949–60.
- Falcioni R, Antonini A, Nistico P et al.  $\alpha 6 \beta 4$  and  $\alpha 6 \beta 1$  integrins associate with ErbB-2 in human carcinoma cell lines. *Exp Cell Res* 1997; 236: 76–85.
- Gambaledda D, Marchetti A, Benedetti L et al. Cooperative signaling between  $\alpha 6 \beta 4$  integrin and ErbB-2 receptor is required to promote phosphatidylinositol 3-kinase-dependent invasion. *J Biol Chem* 2000; 275: 10604–10.
- Shaw LM. Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the  $\alpha 6 \beta 4$  integrin-dependent activation of phosphoinositide 3-OH kinase and promotion of invasion. *Mol Cell Biol* 2001; 21: 5082–93.
- Dans M, Gagnoux-Palacios L, Blaikie P et al. Tyrosine phosphorylation of the  $\beta 4$  integrin cytoplasmic domain mediates Shc signaling to extracellular signal-regulated kinase and antagonizes formation of hemidesmosomes. *J Biol Chem* 2001; 276: 1494–502.
- Mainiero F, Pepe A, Yeon M et al. The intracellular functions of  $\alpha 6 \beta 4$  integrin are regulated by EGF. *J Cell Biol* 1996; 134: 241–53.
- O'Connor KL, Nguyen BK, Mercurio AM. RhoA function in lamellae formation and migration is regulated by the  $\alpha 6 \beta 4$  integrin and cAMP metabolism. *J Cell Biol* 2000; 148: 253–8.
- Jauliac S, Lopez-Rodriguez C, Shaw LM et al. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. *Nat Cell Biol* 2002; 4: 540–4.
- Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002; 296: 550–3.
- Paddison PJ, Caudy AA, Bernstein E et al. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* 2002; 16: 948–58.
- Sui G, Soohoo C, Affar EB et al. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA* 2002; 99: 5515–20.
- Reznicek GA, de Pereda JM, Reipert S, Wiche G. Linking integrin  $\alpha 6 \beta 4$ -based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the  $\beta 4$  subunit and plectin at multiple molecular sites. *J Cell Biol* 1998; 141: 209–25.
- Hintermann E, Bilban M, Sharabi A, Quaranta V. Inhibitory role of  $\alpha 6 \beta 4$ -associated erbB-2 and phosphoinositide 3-kinase in keratinocyte haptotactic migration dependent on  $\alpha 3 \beta 1$  integrin. *J Cell Biol* 2001; 153: 465–78.
- Nguyen BP, Gil SG, Carter WG. Deposition of laminin 5 by keratinocytes regulates integrin adhesion and signaling. *J Biol Chem* 2000; 275: 31896–907.
- McCaffrey AP, Meuse L, Pham TT et al. RNA interference in adult mice. *Nature* 2002; 418: 38–9.
- Lewis DL, Hagstrom JE, Loomis AG et al. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat Genet* 2002; 32: 107–8.
- Brummelkamp T, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* 2002; 2: 243.

## Competing Autocrine Pathways Involving Alternative Neuropilin-1 Ligands Regulate Chemotaxis of Carcinoma Cells<sup>1</sup>

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### Abstract

Neuropilin-1 (NP1), in conjunction with plexins, promotes axon repulsion by binding to semaphorin 3A (SEMA3A). Although NP1 is expressed in carcinoma cells, its functions have remained elusive, and neither SEMA3A nor plexin expression has been explored in cancer. Here we provide evidence that breast carcinoma cells support an autocrine pathway involving SEMA3A, plexin-A1, and NP1 that impedes their ability to chemotax. Reducing SEMA3A or NP1 expression by RNA interference or inhibiting plexin-A1 signaling enhanced migration. Conversely, expression of constitutively active plexin-A1 impaired chemotaxis. The paradox of how breast carcinoma cells expressing these endogenous chemotaxis inhibitors are able to migrate is explained by their expression of vascular endothelial growth factor (VEGF), a NP1 ligand that competes with SEMA3A for receptor binding. Finally, we establish that the ratio of endogenous VEGF and SEMA3A concentrations in carcinoma cells determines their chemotactic rate. Our findings lead to the surprising conclusion that opposing autocrine loops involving NP1 regulate the chemotaxis of breast carcinoma cells. Moreover, our data indicate a novel autocrine function for VEGF in chemotaxis.

### Introduction

In addition to the classical VEGF<sup>3</sup> tyrosine kinase receptors, KDR and Flt-1, NP1 serves as a high-affinity VEGF receptor (1). NP1 expression on endothelial cells enhances VEGF signaling by increasing the affinity of VEGF for the classical VEGF receptor tyrosine kinase KDR (1). Interestingly, NP1 expression has also been reported in a variety of tumors in the absence of KDR or Flt-1 (1, 2). On the basis of the established importance of VEGF in tumor progression, our previous studies investigated a role for NP1 in carcinoma cells as a VEGF receptor, in the absence of classical VEGF receptor tyrosine kinases. These studies indicated that NP1 supports a VEGF autocrine signaling pathway that is critical for breast carcinoma cell survival (2).

Of note, NP1 was identified originally in neurons as a receptor for SEMA3A, a soluble member of the semaphorin family that plays a critical role in axon guidance (3, 4). The ability of NP1, which lacks consensus signaling domains, to deliver SEMA3A-associated chemorepulsive signals is dependent on NP1 associations with plexins, proteins displaying Met homologies (5, 6). Although functions for

NP1 as a VEGF receptor in tumor cells have been reported (2, 7), the possibility that NP1 influences tumor function by supporting signaling through its alternative ligand, SEMA3A, has not been examined. Here, we provide the first evidence for expression of SEMA3A and plexin-A1 in carcinoma cells and demonstrate that these molecules are autocrine inhibitors of breast carcinoma migration. Importantly, we also identify a novel function for VEGF in carcinoma cell migration involving its inhibition of SEMA3A activity.

### Materials and Methods

**mRNA Detection.** mRNA was purified from the indicated cell lines using the RNEasy kit (Qiagen) according to the manufacturer's recommended protocol. RNA (2 µg) was added to RT-PCR reactions containing the indicated primers at a concentration of 0.6 µM. Alternatively, cDNA was generated from carcinoma cells purified from human breast tumors (provided by K. Polyak, Dana-Farber Cancer Institute). The conditions for amplifying SEMA3A and NP1 cDNA were as follows: 35 cycles, 95°C, 15 min; 95°C, 30 s; 55°C, 1 min; and 72°C, 1 min, followed by a 72°C, 10-min final extension step. The conditions for amplifying plexin-A1 cDNA were as follows: 35 cycles, 95°C, 15 min; 95°C, 30 s; 58°C, 1 min; and 72°C, 2.5 min, followed by a 72°C, 10-min extension step. The sequences of amplification primers are as follows: SEMA3A Forward, GACTTTGCTATCTTCCGAACCTTGGGCAC; SEMA3A Reverse, GCTATACATACACACGGCTGATCCCTTG; NP1 Forward, ATGGAGAGGGGGCTGCCG; NP1 Reverse, CTATCGCGCTGTGCGGTGTA; Plexin-A1 Forward, GAGGATGCCGACATGTTCCGGCTTCG; and Plexin-A1 Reverse, AGGGCGTCATGGGCACGCACGG.

**RNAi Transient Transfections.** RNAis were designed and synthesized by Dharmacon, Inc. (see below for sequences). Cells at 60% confluency were transfected in penicillin/streptomycin-free medium with the indicated RNAi using TKO lipid (Mirus), following the manufacturer's recommended protocol. The following RNAi concentrations were determined to be optimal for inhibiting protein expression: 200 nM RNAi for all cell lines; 200 nM SEMA3A RNAi for MDA-231 cells; 100 nM SEMA3A RNAi for MDA-435 and MCF-7 cells. After 20 h, RNAis were removed, and the cells were maintained in complete medium with the indicated antibodies for an additional 24 h: NP1 RNAi, GAGAGGUCCUGAAUGUUCCTT; Scrambled NP1 control, AGAUGAUGUCGUCGUCCTT; SEMA3A RNAi: AAAGUUCUUAGUGCCACCU; and Scrambled SEMA3A control, AAGUGCACGCCUUAUAUAUC.

**SEMA3A and SCR SEMA3A RNAi Retrovirus Generation.** To create SEMA3A-pSUPER and SCR SEMA3A-pSUPER expression vectors, the following oligonucleotides (Invitrogen, Grand Island, NY) were cloned into pSUPER (a gift from R. Agami, The Netherlands Cancer Institute, Amsterdam, the Netherlands): SEMA3A, 5'-gatccccAGTTCATTAGTGCCACCTTcaagagaAGGTGGGCACTAATGAACTttttggaaa-3' and 5'-agcttttccaaaaAGTTCATTAGTGCCACCTTctcttgaaAGGTGGGCACTAATGAACTggg-3'; SCR SEMA3A, 5'-gatccccGTGCACGCCTCTATATATCtcaagagaGATATATAGAGCGTGACctttttggaaa-3' and 5'-agcttttccaaaaGTGCACGCCTCTATATATCtcttgaaGATATATAGAGCGTGACggg-3'. EcoRI- and XhoI-digested inserts containing the H1-RNA promoter and targeting oligonucleotides from pSUPER were then subcloned into pSUPER.retro (Oligoengine, Seattle, WA). All plasmids were sequenced to confirm that they were correct.

To generate retroviruses, SEMA3A-pSUPER or SCR SEMA3A-pSUPER.

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<sup>3</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; NP1, neuropilin-1; SEMA3A, semaphorin 3A; RNAi, RNA interference; ZVAD-FMK, benzylloxycarbonyl-VAD-fluoromethyl ketone; β-gal, β-galactosidase; VSV, vesicular stomatitis virus; AS, antisense.

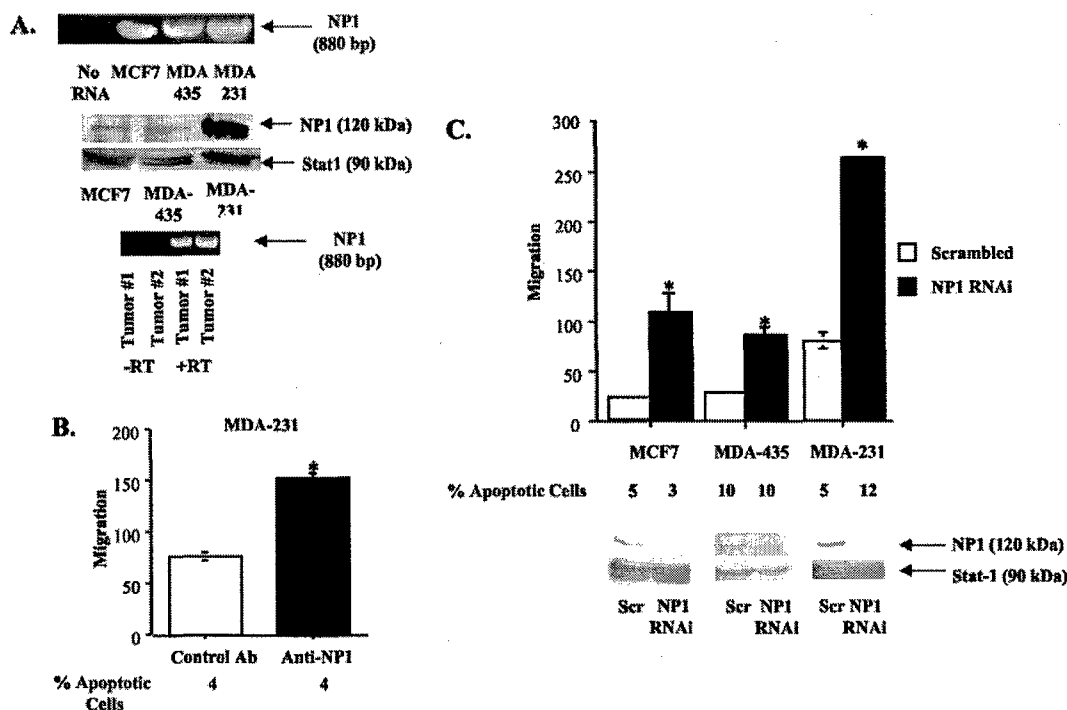


Fig. 1. NP1 suppresses chemotaxis of breast carcinoma cells. **A:** top panel, NP1-specific RT-PCR reactions were performed using the indicated mRNAs; middle panel, protein extracts were immunoblotted with the indicated antibodies (mouse anti-NP1 and rabbit anti-Stat1; Santa Cruz Biotechnology); bottom panel, NP1 was PCR amplified from cDNA obtained from carcinoma cells isolated from breast tumors. **B:** MDA-231 cells were incubated for 6 h (+ ZVAD-FMK) with either a rabbit IgG or NP1-specific polyclonal antibody (Ab; provided by Alex Kolodkin, Johns Hopkins University School of Medicine, Baltimore, MD), and their chemotaxis toward conditioned 3T3 medium was assessed in a 3-h assay in the continued presence of antibody (+ ZVAD-FMK). **C:** cells were transfected with a scrambled (Scr) or NP1-specific RNAi, and their ability to migrate toward conditioned 3T3 medium was assessed in a 24-h (MCF7), 15-h (MDA-435), or 3-h (MDA-231) assay. The mean number (bars,  $\pm$ SD) of migrated cells from two wells (four fields/well) is indicated. \*,  $P < 0.02$  in a Student's  $t$  test. Inhibition of NP1 expression by NP1 RNAi was assessed by immunoblotting, as described in A. The percentage of apoptotic cells was assessed using Annexin V-FITC, as described previously (17). Similar results were observed in three independent experiments.

retro and expression plasmids containing proteins required for viral propagation (Imgenex, San Diego, CA) were transfected into 293T cells. Viral supernatants were harvested, and MDA-MB-435 recipient cells were infected in the presence of 8  $\mu$ g/ml of Polybrene (Sigma, St. Louis, MO). After infection for 24 h, resistant cells were selected with puromycin (2  $\mu$ g/ml).

**DNA Transfections.** Cells were transfected in the presence of Lipofectamine (Life Technologies, Inc.) and ZVAD-FMK with a  $\beta$ -gal-expressing plasmid (1  $\mu$ g), and either VSV-tagged, dominant-negative human plexin-A1 (plexin-A1 $\Delta$ Cyt, provided by P. Comoglio, University of Torino, Italy) or myc-tagged constitutively active murine plexin-A1 (1  $\mu$ g of PlexA1 $\Delta$ Sem, provided by S. Strittmatter, Yale University School of Medicine, New Haven, CT). The ability of these transfectants to migrate toward conditioned medium was assessed after 48 h in the presence of ZVAD-FMK.

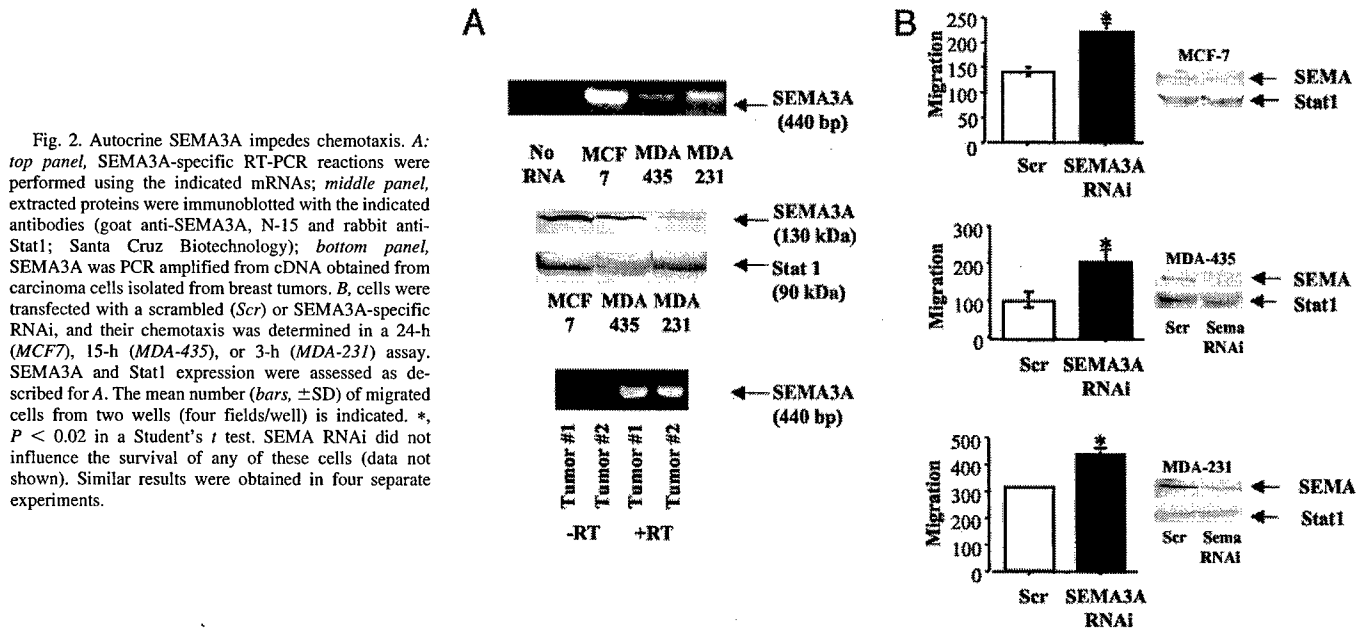
**Chemotaxis Assays.** Chemotaxis toward conditioned NIH3T3 medium was assessed using collagen (Cohesion; 15  $\mu$ g/ml)-coated Transwell chambers, as described previously (8).

## Results and Discussion

Given that NP1 is expressed in breast carcinoma cell lines (Refs. 1 and 2; Fig. 1A) and tumors (Fig. 1A), we assessed the potential involvement of this receptor in carcinoma chemotaxis. Surprisingly, a NP1-neutralizing antibody increased the chemotaxis of MDA-231 cells toward NIH 3T3 conditioned medium 2-fold (Fig. 1B). To confirm and extend this finding, we implemented an RNAi strategy to diminish NP1 expression in each of three breast carcinoma cell lines. Our previous data indicate that NP1 is essential for breast carcinoma survival because it supports VEGF autocrine survival signaling (2). To evaluate the role of NP1 in migration separately from its requirement for breast carcinoma cell survival (2), NP1 RNAi transfections were performed in the presence of the general caspase inhibitor, ZVAD-FMK. Under these conditions, the inhibition of NP1 expres-

sion did not impact cell survival (Fig. 1C). Of note, this RNAi abolished NP1 expression in MDA-435 and MDA-231 cells, and it increased their chemotaxis by 1.5- and 2.3-fold, respectively (Fig. 1C). In addition, this RNAi reduced NP1 expression in MCF-7 cells, a poorly migratory breast carcinoma line, and enhanced their chemotaxis 5-fold (Fig. 1C).

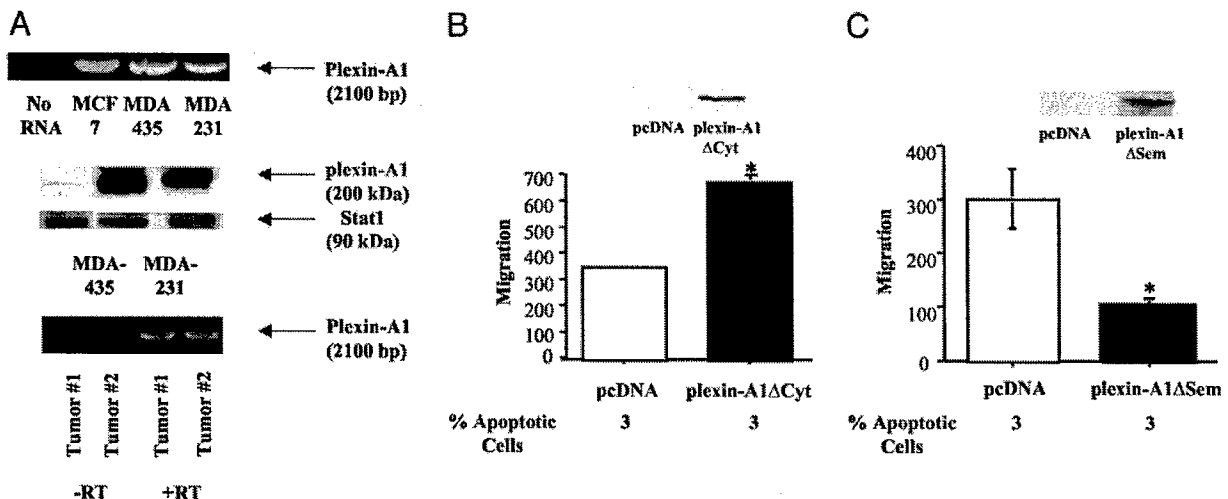
SEMA3A inhibits axon outgrowth by binding to NP1 and the NP1 coreceptor, plexin-A1 (5, 6). On the basis of our finding that NP1 is inhibitory for breast carcinoma migration, we hypothesized that these cells express SEMA3A and plexin-A1. In fact, SEMA3A and plexin-A1 mRNA were detected in each of three breast carcinoma cell lines, as well as in primary breast tumors (Figs. 2A and 3A). We also identified SEMA3A and plexin-A1 protein by immunoblotting proteins extracted from these samples with a SEMA3A- or plexin-A1-specific antibody (Figs. 2A and 3A). To elucidate a function for SEMA3A in breast carcinoma cells, we reduced SEMA3A expression using a SEMA3A RNAi. This RNAi, which reduced SEMA3A expression significantly (Fig. 2B), increased the migration of these cells (Fig. 2B) without influencing cell survival (data not shown). To assess the importance of plexin-A1 in migration, MDA-231 cells were transfected with a plexin-A1 cytoplasmic domain deletion mutant that inhibits SEMA3A signaling (6). Expression of this mutant in MDA-231 cells enhanced their migration significantly (Fig. 3B). Conversely, expression of a semaphorin homology domain deletion mutant of plexin-A1 that exhibits constitutive activity in neurons (9) inhibited MDA-231 migration (Fig. 3C). None of these reagents influenced cell survival in the presence of ZVAD-FMK (Fig. 3, B and C). These data indicate that an autocrine pathway involving



SEMA3A, NP1, and plexin-A1 impedes the chemotaxis of breast carcinoma cells. Our ability to increase breast carcinoma migration by expressing a dominant-negative plexin-A1 suggests that other plexins, if expressed in these cells, cannot support SEMA3A signaling in the absence of plexin-A1 function.

Genes that are inhibitory for cell growth are frequently subject to chromosomal deletion, mutational inactivation, or gene silencing in tumor cells (10–12). The ability of breast carcinoma cells to migrate and invade, despite their expression of molecules involved in SEMA3A signaling, suggested that they support a novel mechanism for repressing SEMA3A function. Increased VEGF expression is a hallmark of breast carcinoma progression (13, 14). Until recently, the function of VEGF in tumor progression was thought to relate solely to its angiogenic activity. We were intrigued by the reported ability of

recombinant VEGF and recombinant SEMA3A, which exhibit similar affinities for NP1 and NP1/plexin complexes, respectively (1, 5), to compete for NP1 binding (15, 16). On the basis of these findings, we postulated that endogenous VEGF and SEMA3A compete for NP1 binding, and that the ratio of the concentration of these proteins in carcinoma cells is a critical determinant of their chemotactic rate. To determine this ratio, we measured the relative amounts of SEMA3A and VEGF protein in these cells (Fig. 4A). We then compared the ratio of these concentrations to the relative chemotactic rate of these carcinoma cells. As shown in Fig. 4B, MCF-7 cells, which exhibited the lowest chemotactic rate, displayed the highest ratio of SEMA3A to VEGF protein. MDA-435 cells, which were more chemotactic than MCF-7 cells, demonstrated a lower SEMA3A:VEGF concentration ratio (Fig. 4B). The lowest SEMA3A:VEGF ratio was observed in



**Fig. 3. Plexin-A1 signaling inhibits chemotaxis.** *A: top panel*, plexin-A1-specific RT-PCR reactions were performed using the indicated mRNAs; *middle panel*, plexin-A1 and Stat1 proteins were detected by immunoblotting (rat anti-plexin-A1 provided by H. Fujisawa, Nagoya University Graduate School of Science, Nagoya, Japan; rabbit anti-Stat1 provided by Santa Cruz Biotechnology); *bottom panel*, plexin-A1 fragments were amplified from cDNA isolated from carcinoma cells purified from breast tumors. *B*, MDA-231 cells were cotransfected with a  $\beta$ -gal-expressing plasmid in addition to either a control plasmid or a VSV-tagged plexin-A1 cytoplasmic domain deletion mutant (*plexin-A1ΔCyt*), and their migration was measured after 48 h by 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside staining. Expression of the VSV-tagged construct was confirmed by immunoblotting with a VSV-specific antibody (Sigma). *C*, MDA-231 cells were cotransfected with a  $\beta$ -gal-expressing vector and either a control plasmid or a constitutively active, myc-tagged plexin-A1 construct (*plexin-A1ΔSem*). The number of migrated cells after 48 h was assessed by 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside staining. Expression of the myc-tagged construct was confirmed by immunoblotting with a myc-specific antibody (Sigma). For *B* and *C*, the mean number (bars,  $\pm$ SD) of migrated cells from two wells (four fields/well), as well as the percentage of apoptotic cells, is indicated. \*,  $P < 0.02$  in a Student's *t* test. Similar results were obtained in two trials.

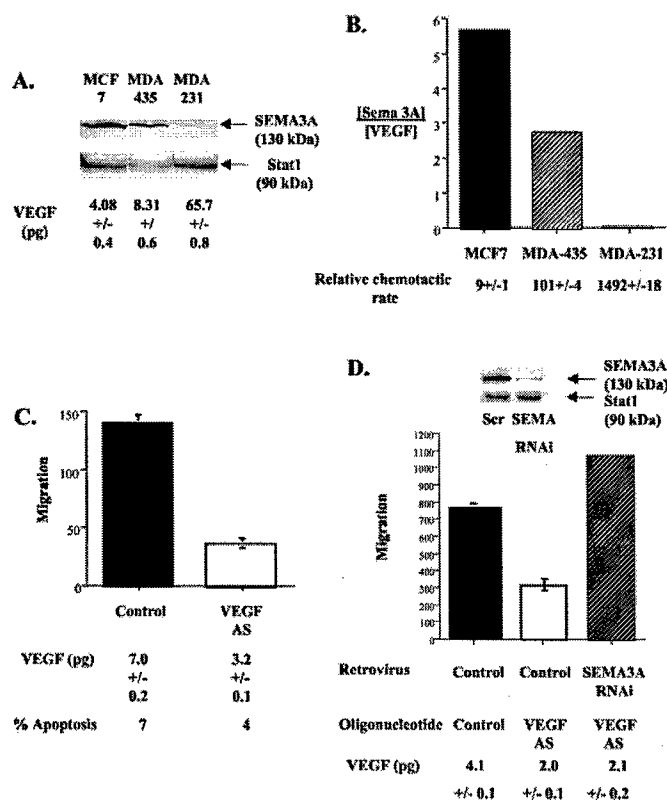


Fig. 4. Relative concentration of VEGF and SEMA3A determines chemotaxis rate. A, SEMA3A expression in equivalent amounts of total cellular protein extracted from MCF7, MDA-435, and MDA-231 cells was assessed by immunoblotting and quantified by densitometry. VEGF expression (pg) in 100  $\mu$ g of total proteins extracted from these cells was measured by ELISA (Research and Diagnostic Systems). B, the ratio of relative levels of SEMA3A and VEGF was determined for the indicated breast carcinoma cell lines. In addition, the rates of chemotaxis for these carcinoma lines were assessed by determining the mean number of cells (bars,  $\pm$ SD) that had migrated toward conditioned medium in a 16-h assay. Similar trends were observed in three trials. C, MDA-435 cells were transfected with either a VEGF AS or sense (Control) oligonucleotide as described previously (Ref. 2; + ZVAD-FMK), and their migration toward conditioned medium was assessed in a 15-h assay. The percentage of apoptotic cells was measured as described for Fig. 1. D, MDA-435 cells were infected stably with either a SEMA3A RNAi-expressing or control retrovirus. These retroviral cells were then transfected transiently with either a control or VEGF AS oligonucleotide (+ ZVAD-FMK). The ability of these cells to migrate toward conditioned medium was determined in a 15-h assay. For C and D, the mean number (bars,  $\pm$ SD) of migrated cells from two wells (four fields/well) was determined. \*,  $P < 0.02$  in a Student's  $t$  test. VEGF and SEMA3A expression were assessed as described for A. Similar results were obtained in two separate experiments.

MDA-231 cells, which exhibited the most robust rate of chemotaxis (Fig. 4B).

If the relative concentrations of endogenous VEGF and SEMA3A in breast carcinoma cells determine their chemotactic rate, then altering expression of these NPI ligands should influence chemotaxis. To reduce VEGF expression, MDA-435 cells were transfected with either a VEGF AS or control oligonucleotide. VEGF AS transfection reduced VEGF expression by ~50% relative to transfection with the control oligonucleotide (Fig. 4C). Importantly, VEGF AS transfection did not reduce SEMA3A expression in these cells (data not shown). Strikingly, the ability of VEGF AS transfectants to migrate toward conditioned medium was reduced relative to that of the control transfectants (Fig. 4C). These transfectants were maintained in the presence of ZVAD-FMK, and the reduction in VEGF expression did not influence their survival (Fig. 4C).

If a major role for VEGF in carcinoma cells involves its antagonism of autocrine SEMA3A, then reducing SEMA3A expression in VEGF AS transfectants should offset the decrease in chemotaxis caused by reduced VEGF expression. To decrease SEMA3A expression stably,

MDA-435 cells were infected with retroviruses expressing either a SEMA3A-specific or scrambled RNAi (control). Stable infectants that expressed SEMA3A RNAi exhibited a significant decrease in SEMA3A expression relative to cells infected with the control retrovirus (Fig. 4D). These infectants were then transfected transiently with either the VEGF AS or control oligonucleotide. Confirming the data in Fig. 4C, VEGF expression in VEGF AS transfectants was reduced by 50%. We then determined the ability of these cells to chemotax toward conditioned NIH 3T3 medium. Confirming the data in Fig. 4C, the chemotaxis of cells infected with the control retrovirus was significantly reduced by VEGF AS transfection. Strikingly, the migration of VEGF AS transfectants was restored upon reducing SEMA3A expression with the SEMA3A RNAi-expressing retrovirus. In the presence of ZVAD-FMK, we did not observe an effect of reducing either SEMA3A or VEGF expression on cell survival (data not shown). These data identify VEGF and SEMA3A as antagonistic, autocrine NPI ligands that regulate breast carcinoma migration.

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## References

- Soker, S., Takshima, S., Miao, H., Neufeld, G., and Klagsbrun, M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell*, 92: 735-745, 1998.
- Bachelder, R. E., Crago, A., Chung, J., Wendt, M. A., Shaw, L. M., Robinson, G., and Mercurio, A. M. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res.*, 61: 5736-5740, 2001.
- He, Z., and Tessier-Lavigne, M. Neuropilin is a receptor for the axonal chemorepellent semaphorin III. *Cell*, 90: 739-751, 1997.
- Kolodkin, A. L., Levengood, D. V., Rowe, E., Tai, Y., Giger, R. J., and Ginty, D. D. Neuropilin is a semaphorin III receptor. *Cell*, 90: 753-762, 1997.
- Takahashi, T., Fournier, A., Nakamura, F., Wang, L., Murakami, Y., Kalb, R. G., Fujisawa, H., and Strittmatter, S. M. Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell*, 99: 59-69, 1999.
- Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G., Song, H., Chedotal, A., Winberg, M. L., Goodman, C. S., Poo, M., Tessier-Lavigne, M., and Comoglio, P. M. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell*, 99: 71-80, 1999.
- Miao, H., Lee, P., Lin, H., Soker, S., and Klagsbrun, M. Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. *FASEB J.*, 14: 2532-2539, 2000.
- Shaw, L. M., Rabinovitz, I., Wang, H., Toker, A., and Mercurio, A. M. Activation of phosphoinositide 3-OH kinase by the  $\alpha 6 \beta 4$  integrin promotes carcinoma invasion. *Cell*, 91: 949-960, 1997.
- Takahashi, T., and Strittmatter, S. M. PlexinA1 autoinhibition by the plexin SEMA domain. *Neuron*, 29: 429-439, 2001.
- Cho, K. R., and Vogelstein, B. Genetic alterations in the adenoma-carcinoma sequence. *Cancer (Phila.)*, 70: 1727-1731, 1992.
- Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., and Bos, J. L. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.*, 19: 525-532, 1988.
- Herman, J. G. Hypermethylation of tumor suppressor genes in cancer. *Semin. Cancer Biol.*, 9: 359-367, 1999.
- Brown, L. F., Guidi, A. J., Schnitt, S. J., Van de Water, L., Iruela-Arispe, M. L., Yeo, T. K., Tognazzi, K., and Dvorak, H. F. Vascular stroma formation in carcinoma *in situ*, invasive carcinoma, and metastatic carcinoma of the breast. *Clin. Cancer Res.*, 5: 1041-1056, 1999.
- Salgen, P., Perhoniemi, V., Tykka, H., Maenpaa, H., and Joensuu, H. Serum VEGF levels in women with a benign breast tumor or breast cancer. *Breast Cancer Res. Treat.*, 53: 161-166, 1999.
- Miao, H., Soker, S., Feiner, L., Alonso, J. L., Raper, J. A., and Klagsbrun, M. Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. *J. Cell Biol.*, 146: 233-241, 1999.
- Bagnard, D., Vaillant, C., Khuth, S., Dufay, N., Lohrum, M., Puschel, A. W., Belin, M., Bolz, J., and Thomasset, N. Semaphorin 3A-vascular endothelial growth factor-165 balance mediates migration and apoptosis of neural progenitor cells by the recruitment of shared receptor. *J. Neurosci.*, 21: 3332-3341, 2001.
- Bachelder, R. E., Ribick, M. J., Marchetti, A., Falcioni, R., Soddu, S., Davis, K. R., and Mercurio, A. M. p53 inhibits  $\alpha 6 \beta 4$  integrin survival signaling by promoting the caspase 3-dependent cleavage of AKT/PKB. *J. Cell Biol.*, 147: 1063-1072, 1999.

# Integrin ( $\alpha 6 \beta 4$ ) regulation of eIF-4E activity and VEGF translation: a survival mechanism for carcinoma cells

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**W**e define a novel mechanism by which integrins regulate growth factor expression and the survival of carcinoma cells. Specifically, we demonstrate that the  $\alpha 6 \beta 4$  integrin enhances vascular endothelial growth factor (VEGF) translation in breast carcinoma cells. The mechanism involves the ability of this integrin to stimulate the phosphorylation and inactivation of 4E-binding protein (4E-BP1), a translational repressor that inhibits the function of eukaryotic translation initiation factor 4E (eIF-4E). The regulation of 4E-BP1 phosphorylation by  $\alpha 6 \beta 4$  derives from the ability of this integrin to activate the PI-3K-Akt

pathway and, consequently, the rapamycin-sensitive kinase mTOR that can phosphorylate 4E-BP1. Importantly, we show that this  $\alpha 6 \beta 4$ -dependent regulation of VEGF translation plays an important role in the survival of metastatic breast carcinoma cells by sustaining a VEGF autocrine signaling pathway that involves activation of PI-3K and Akt. These findings reveal that integrin-mediated activation of PI-3K-Akt is amplified by integrin-stimulated VEGF expression and they provide a mechanism that substantiates the reported role of  $\alpha 6 \beta 4$  in carcinoma progression.

## Introduction

An understanding of the mechanisms that sustain the survival of tumor cells in adverse physiological conditions is one of the most important problems in cancer biology. As argued recently, cancer progression is an evolutionary process that selects for cells that exhibit the capacity for survival in environmental conditions not present in normal tissue (Fearon, 1999; Hanahan and Weinberg, 2000). One survival strategy used by tumor cells is the secretion of proteins that elicit an angiogenic response, such as vascular permeability factor or vascular endothelial growth factor (VEGF).<sup>\*</sup> VEGF appears to be an essential factor for the progression of many solid tumors (Shweiki et al., 1992; Brown et al., 1999; Dvorak et al., 1999). It is widely assumed that the function of VEGF produced by tumor and tumor stromal cells is to stimulate angiogenesis by acting in a paracrine fashion on vicinal endothelium (Hanahan and Folkman, 1996; Brown et al., 1999). Another mechanism for tumor cell survival is the establishment of

autocrine signaling loops that act on tumor cells directly (Scotlandi et al., 1996; Tokunou et al., 2001; Wong et al., 2001). Although the significance of this mechanism has been overshadowed by angiogenesis, recent studies have substantiated the importance and necessity of such signaling loops for tumor survival (Scotlandi et al., 1996; Bachelder et al., 2001; Tokunou et al., 2001; Wong et al., 2001). Indeed, this mechanism probably contributes to the ability of cells to survive in hypoxic, poorly vascularized regions of tumors. In this direction, we described recently the existence of a VEGF autocrine signaling pathway in metastatic breast carcinoma cells that is essential for their survival (Bachelder et al., 2001).

An important issue that arises from the contribution of VEGF autocrine signaling to tumor survival is an understanding of the mechanisms that regulate VEGF expression. Such mechanisms are important not only for VEGF signaling in tumor cells, but also for angiogenesis as well. Clearly, hypoxia is a strong inducer of VEGF transcription and mRNA stability (von Marschall et al., 2001), but other factors are likely to be involved. Of note, our finding that the  $\alpha 6 \beta 4$  integrin can promote the survival of breast carcinoma cells in stress conditions is intriguing (Bachelder et al., 1999b) and raised the novel possibility that a specific integrin, which has been implicated in cancer progression, could regulate VEGF expression. This possibility is substantiated by the finding reported here that the ability of the  $\alpha 6 \beta 4$  integrin to promote survival is VEGF dependent.

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<sup>\*</sup>Abbreviations used in this paper: 4E-BP1, 4E-binding protein; eIF-4E, eukaryotic initiation factor-4E; mTOR, mammalian target of rapamycin; Myr-Akt, myristoylated Akt; PI, propidium iodide; PI-3K, phosphatidylinositol 3-kinase; RNAi, small interfering RNA; VEGF, vascular endothelial growth factor.

Key words: integrin; VEGF; translation; carcinoma; eIF-4E

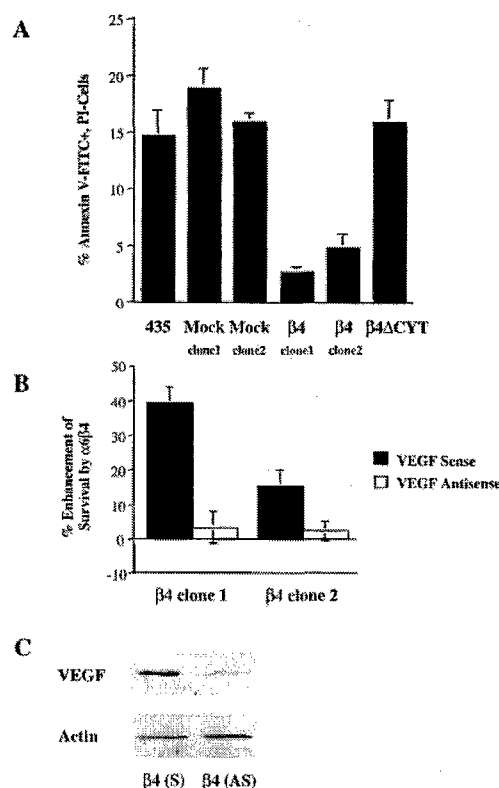


The results described above prompted us to investigate the relationship between the  $\alpha 6\beta 4$  integrin and VEGF expression. We observed that the expression and signaling properties of this integrin have no impact on steady-state VEGF mRNA levels. Surprisingly, however, we detected a significant influence of  $\alpha 6\beta 4$  on VEGF translation and protein expression in these cells, an observation that reveals the ability of this integrin to regulate translation. The mechanism by which  $\alpha 6\beta 4$  regulates VEGF expression involves its ability to stimulate the phosphorylation of 4E-binding protein (4E-BP1). 4E-BP1 is phosphorylated by mammalian target of rapamycin (mTOR), a protein kinase whose catalytic domain is structurally related to that of phosphatidylinositol 3-kinase (PI-3K) (Dennis et al., 1999; Schmelzle and Hall, 2000). Phosphorylation of 4E-BP1 by mTOR disrupts its binding to eukaryotic translation initiation factor eIF-4E, which is present in rate-limiting amounts in most cells (De Benedetti and Harris, 1999; McKendrick et al., 1999). eIF-4E plays a critical role in the recruitment of the translational machinery to the 5' end of mRNA, which is demarcated by an m7GpppN cap (where m is a methyl group and N is any nucleotide) (Raught and Gingras, 1999). The m7 cap is essential for the translation of most mRNAs including VEGF (De Benedetti and Harris, 1999; Raught and Gingras, 1999). Dissociation of 4E-BP1 from eIF-4E enables eIF-4E to initiate translation (Gingras et al., 1999, 2001b). The regulation of 4E-BP1 phosphorylation by  $\alpha 6\beta 4$  derives from the ability of this integrin to activate the PI-3K-Akt pathway and, consequently, mTOR. Our findings reveal a novel mechanism of tumor cell survival and they highlight the ability of a specific integrin to regulate protein translation by influencing eIF-4E activity.

## Results

### The ability of the $\alpha 6\beta 4$ integrin to promote the survival of carcinoma cells is VEGF dependent

To examine the hypothesis that the ability of the  $\alpha 6\beta 4$  integrin to promote survival is VEGF dependent, we used MDA-MB-435 cells, which lack expression of this integrin. Stable expression of  $\alpha 6\beta 4$  in these cells enhances their ability to survive in stressful conditions (Bachelder et al., 1999b). Importantly, however, expression of  $\alpha 6\beta 4$  does not alter the expression of other integrin subunits in these cells and does not influence their adhesion to matrix (Shaw et al., 1997). As shown in Fig. 1 A, a significant level of apoptosis was observed after 24 h of serum deprivation in the parental MDA-MB-435 cells and mock transfectants, as well as in transfectants that express  $\alpha 6\beta 4$  containing a cytoplasmic domain deletion of the  $\beta 4$  subunit that lacks the ability to signal (Shaw et al., 1997). Stable subclones that express the intact  $\alpha 6\beta 4$  integrin, however, were protected from apoptosis under these conditions. Based on these results and our previous finding that the survival of metastatic breast carcinoma cells is dependent on VEGF, we used a VEGF antisense oligonucleotide to reduce VEGF expression in the MDA-MB-435/ $\beta 4$  transfectants and assessed the impact of reducing VEGF expression on their survival (Fig. 1, B and C). The VEGF antisense oligonucleotide reduced VEGF protein expression significantly in the  $\beta 4$  transfectants (Fig.

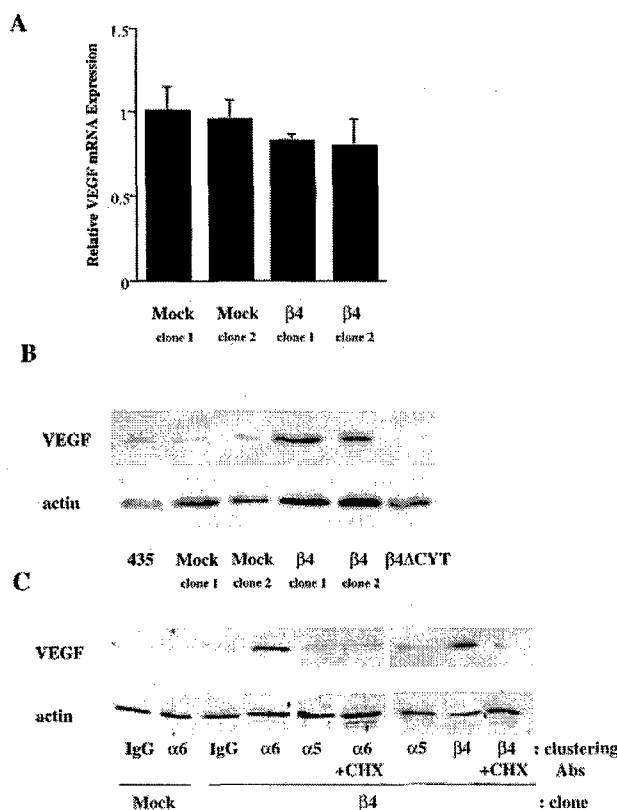


**Figure 1. The  $\alpha 6\beta 4$ -mediated survival of breast carcinoma cells is VEGF dependent.** (A) Parental, mock (clone 1, 6D2; clone 2, 6D7),  $\beta 4$ - $\Delta$ CYT-expressing (cytoplasmic tail deletion mutant), and  $\beta 4$  integrin-expressing (clone 1, 3A7; clone 2, 5B3) MDA-MB-435 subclones were maintained in low serum (0.5% FBS) medium for 24 h. To assess the level of apoptosis, these cells were stained with annexin V-FITC and propidium iodide (PI), and analyzed on a Becton Dickinson flow cytometer using CellQuest software. The percentage of annexin-positive, PI-negative cells ( $\pm$  SD) is indicated. Results were obtained from three independent experiments. Apoptosis was minimal in the presence of 10% FBS (unpublished data). (B) Mock-transfected clone 6D7 and  $\beta 4$  integrin- (clone 1, 3A7; clone 2, 5B3) expressing MDA-MB-435 subclones were transiently transfected with VEGF sense or antisense oligonucleotides and maintained in low serum (0.5% FBS) medium. After 24 h, the level of apoptosis in these cells was assessed as described above. The data are presented as the mean difference ( $\pm$  SD) in annexin positivity between mock-transfected and  $\alpha 6\beta 4$ -expressing MDA-MB-435 cells. Similar results were observed in two separate experiments. (C) The relative amount of VEGF protein in extracts obtained from the MDA-MB-435/ $\beta 4$  cells transfected with either the VEGF sense (S) or antisense (AS) oligonucleotide was determined by immunoblotting using a polyclonal anti-VEGF immune serum.

1 C). As shown in Fig. 1 B, this reduction in VEGF expression abrogated the survival-enhancing effect of  $\alpha 6\beta 4$  under conditions of serum deprivation.

### The $\alpha 6\beta 4$ integrin increases VEGF protein but not mRNA expression

Given that the survival effect of  $\alpha 6\beta 4$  expression is VEGF dependent, the novel possibility arose that VEGF expression could be regulated by this integrin. VEGF expression can be regulated at the level of both transcription and mRNA stability (Nabors et al., 2001; von Marschall et al., 2001), mechanisms that would alter the steady-state levels of VEGF



**Figure 2. Expression of the  $\alpha 6 \beta 4$  integrin increases VEGF protein but not steady-state mRNA.** (A) The amount of VEGF mRNA in extracts obtained from mock- (clone 1, 6D2; clone 2, 6D7) and  $\beta 4$  integrin- (clone 1, 3A7; clone 2, 5B3) transfected MDA-MB-435 subclones was quantified by real-time PCR. The data are presented as the mean ratio of VEGF to  $\beta$ -actin mRNA ( $\pm$  SD) obtained from triplicate samples. (B) Parental (435), mock (clone 1, 6D2; clone 2, 6D7),  $\beta 4$ - $\Delta CYT$ -expressing (clone 1E10), and  $\beta 4$  integrin-expressing (clone 1: 3A7, clone 2: 5B3) MDA-MB-435 subclones were cultured in low serum (0.5% FBS) medium for 24 h. Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. Similar results were observed in four independent experiments. (C) Mock (clone 6D7) and  $\beta 4$  integrin-expressing (clone 3A7) MDA-MB-435 subclones were maintained in low serum (0.5% FBS) medium for 24 h. These cells were detached with trypsin and incubated with integrin-specific antibodies ( $\alpha 6$  integrin, 2B7;  $\beta 4$  integrin, A9;  $\alpha 5$  integrin, Sam1) or IgG for 30 min in suspension and allowed to adhere on anti-IgG-coated plates for 60 min before lysis. In addition, cells were preincubated in cycloheximide (CHX) at a concentration of 10  $\mu$ g/ml for 30 min and then incubated with either the  $\alpha 6$  or  $\beta 4$  integrin antibodies in the presence of cycloheximide. Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. Similar results were observed in two independent experiments.

mRNA. In addition, regulation can also occur at the level of VEGF translation (Kevil et al., 1996; Akiri et al., 1998; Stein et al., 1998). As shown in Fig. 2 A, quantitative analysis of VEGF mRNA levels in two clones of MDA-MB-435/mock and  $\beta 4$  transfectants using real-time PCR revealed no significant difference in the steady-state mRNA levels in these two populations. However, we detected a substantial increase in VEGF protein expression in the MDA-MB-435/ $\beta 4$  transfectants relative to either the parental cells, mock

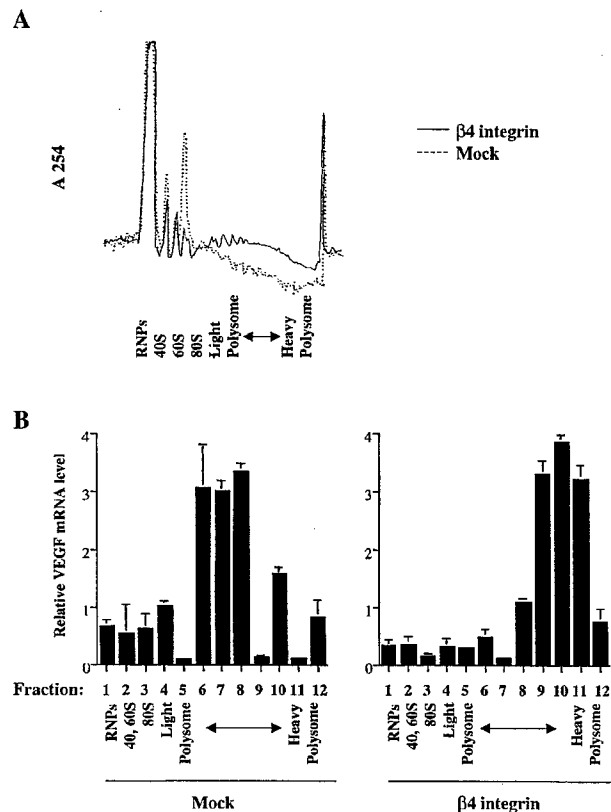
transfectants, or cells that express a cytoplasmic domain deletion of the  $\beta 4$  subunit ( $\beta 4$ - $\Delta CYT$ ) (Fig. 2 B). These results indicate that the  $\alpha 6 \beta 4$  integrin regulates VEGF protein expression. It is also worth noting that the level of apoptosis observed in these populations in response to serum deprivation correlates inversely with their expression of VEGF (Fig. 1 A and Fig. 2 B).

To substantiate the regulation of VEGF expression by  $\alpha 6 \beta 4$ , integrin-specific antibodies were used to cluster either  $\alpha 6 \beta 4$  or  $\alpha 5 \beta 1$  and the effects of integrin-mediated clustering on VEGF expression were assessed by immunoblotting. Of note, the MDA-MB-435/ $\beta 4$  transfectants express equivalent levels of  $\alpha 6 \beta 4$  and  $\alpha 5 \beta 1$  (unpublished data). An  $\alpha 6$ -specific antibody (mAb 2B7) was used to cluster the  $\alpha 6 \beta 1$  integrin in the mock transfectants and the  $\alpha 6 \beta 4$  integrin in the  $\beta 4$  transfectants, a  $\beta 4$ -specific antibody (mAb A9) was used to cluster the  $\alpha 6 \beta 4$  integrin in the  $\beta 4$  transfectants, and an  $\alpha 5$ -specific antibody (mAb Sam1) was used to cluster  $\alpha 5 \beta 1$  in both the mock and  $\beta 4$  transfectants. A substantial induction of VEGF expression was observed upon  $\alpha 6 \beta 4$  integrin clustering in the  $\beta 4$  transfectants but not in the mock transfectants, and no induction was seen in response to  $\alpha 5 \beta 1$  clustering (Fig. 2 C). Importantly, the induction of VEGF expression that occurs in response to  $\alpha 6 \beta 4$  clustering was inhibited by cycloheximide (Fig. 2 C). This result, together with the real-time PCR data (Fig. 2 A), indicates that  $\alpha 6 \beta 4$  is influencing VEGF translation.

To obtain more definitive evidence that  $\alpha 6 \beta 4$  is regulating VEGF translation, we performed polysome analysis of the VEGF message. mRNA isolated from the MDA-MB-435/mock and  $\beta 4$  transfectants was fractionated on a sucrose gradient (Fig. 3 A) and the relative amount of VEGF mRNA in each fraction was determined by real-time PCR (Fig. 3 B). As shown in Fig. 3 B, a striking difference in the distribution of VEGF mRNA was evident in the two populations of cells. In the MDA-MB-435/ $\beta 4$  transfectants, VEGF mRNA fractionated in the heavy polysomal region, whereas in the mock transfectants, the majority of VEGF mRNA was associated with light polysomal to ribosomal subunit fractions. This result indicates that the translation of VEGF in the MDA-MB-435/ $\beta 4$  transfectants is cap dependent.

### Identification of an $\alpha 6 \beta 4$ integrin-mediated signaling pathway that regulates VEGF expression

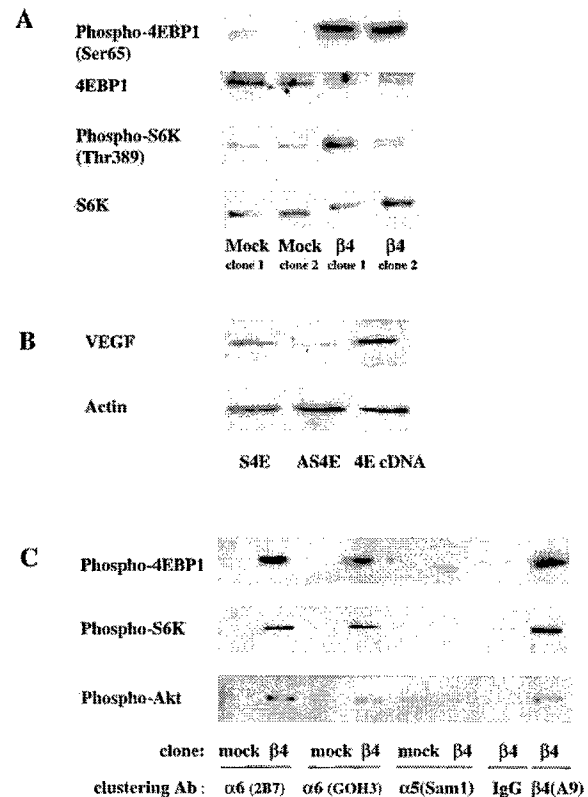
Our finding that  $\alpha 6 \beta 4$  regulates the cap-dependent translation of VEGF prompted us to assess the ability of this integrin to stimulate the activity of the eIF-4E translation initiation factor. The  $\alpha 6 \beta 4$  integrin is a potent activator of the PI-3K-Akt signaling pathway in MDA-MB-435 and other carcinoma cells (Shaw et al., 1997; Bachelder et al., 1999a; Gambaletta et al., 2000; Nguyen et al., 2000; Hintermann et al., 2001), and this pathway has been linked to the regulation of protein translation. Specifically, the serine/threonine kinase mTOR is activated by Akt-mediated phosphorylation events (Sekulic et al., 2000). Phosphorylation of 4E-BP1 by mTOR disrupts its binding to eIF-4E, enabling eIF-4E to initiate translation of VEGF and other molecules (De Benedetti and Harris, 1999). We hypothesized, based on this information, that  $\alpha 6 \beta 4$  regulates 4E-BP1 phosphorylation and, as a consequence, VEGF expression. Initially, we as-



**Figure 3. Polysome analysis of VEGF mRNA.** (A) The distribution of RNA from MDA-MB-435/β4 and mock transfectants that had been fractionated on sucrose gradients as described in the Materials and methods was determined by measuring the  $A_{254}$  of each fraction. (B) The relative VEGF mRNA content of each sucrose gradient fraction was measured by real-time PCR as described in the Materials and methods. Fraction 1 contains unbound RNA present in the ribonucleoprotein fraction, fraction 2 contains 40S and 60S monosomes, fraction 3 contains 80S monosomes, fractions 4–7 contain light polysomes, and fractions 8–12 contain heavy polysomes. The data are presented as the mean ratio of VEGF to β-actin mRNA ( $\pm$  SD) obtained from triplicate samples. Similar results were obtained from three independent experiments.

essed the steady-state phosphorylation levels of 4E-BP1 and S6 kinase ( $p70^{S6K}$ ), which are both downstream targets of mTOR, in cells that had been serum deprived for 24 h. Indeed, a marked increase in the level of phosphorylation of 4E-BP1 (on Ser65) and  $p70^{S6K}$  (on Thr389) was evident in the MDA-MB-435/β4 transfectants relative to either the mock transfectants or the parental cells (Fig. 4 A). Phosphorylation of Ser65 of 4E-BP1 has been shown to be critical for dissociation of 4E-BP from eIF-4E (Gingras et al., 2001a). The reduced expression of 4E-BP1 in the β4 transfectants compared with the mock transfectants that is apparent in Fig. 4 A may reflect the possibility that the 4E-BP Ab does not recognize the hyperphosphorylated form of the protein as well as it recognizes the hypophosphorylated form.

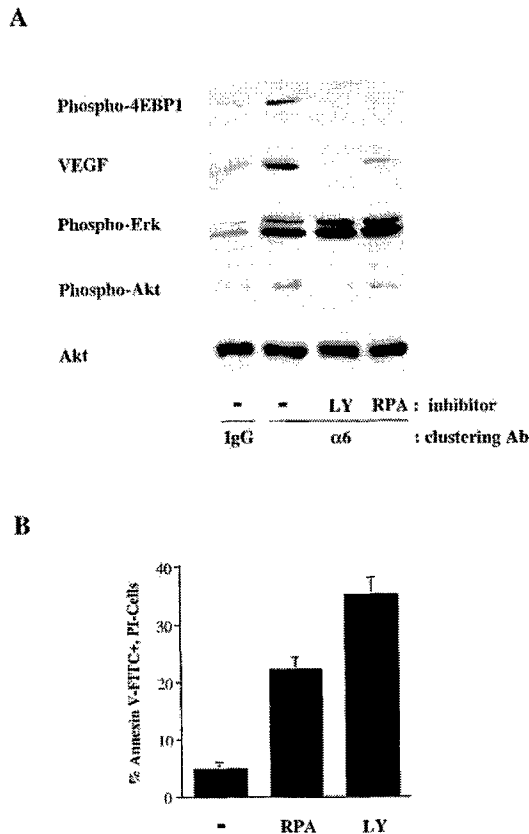
The involvement of eIF-4E in VEGF translation was confirmed by the expression of an antisense eIF-4E oligonucleotide in the MDA-MB-435/β4 transfectants. As shown in Fig. 4 B, expression of this oligonucleotide reduced the level of VEGF protein significantly. In contrast, expression of the full-length eIF-4E cDNA increased the VEGF protein by ap-



**Figure 4. The α6β4 integrin stimulates the phosphorylation of Akt, 4E-BP1, and  $p70^{S6K}$ .** (A) MDA-MB-435 parental cells, mock transfectants, and β4 transfectants were maintained in medium containing low serum (0.5% FBS) for 24 h. The phosphorylation status of 4E-BP1 on Ser 65 and S6K on Thr 389 was assessed in extracts from these cells using phosphospecific antibodies as described in the Materials and methods. In addition, the total amount of 4E-BP1 and  $p70^{S6K}$  in these extracts was assessed by immunoblotting. (B) The MDA-MB-435/β4 cells were transiently transfected with either an eIF-4E sense (S) or antisense (AS) oligonucleotide, or a full-length eIF-4E cDNA (4E). Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. (C) MDA-MB-435 mock (clone 6D7) and β4 (clone 3A7) transfectants were maintained in low serum (0.5% FBS) medium for 24 h. These cells were detached with trypsin and incubated with integrin-specific antibodies (α6 integrin, 2B7; α6 integrin, G0H3; α5 integrin, Sam1; β4 integrin, A9) or IgG for 30 min as described in the legend to Fig. 2. The phosphorylation status of 4E-BP1 (Ser 65), S6K (Thr 389), and Akt (Ser 473) was assessed in extracts from these cells using phosphospecific antibodies. Similar results were observed in four independent experiments.

proximately twofold. These results, together with the polysome analysis data (Fig. 3), indicate that α6β4 regulates VEGF expression by eIF-4E-mediated, cap-dependent translation.

To confirm the specificity of the α6β4 integrin in mTOR signaling, the effects of integrin-mediated clustering on 4E-BP1 phosphorylation were assessed. A substantial induction of Akt, 4E-BP1, and  $p70^{S6K}$  phosphorylation was observed upon α6β4 integrin clustering in the β4 transfectants but not in the mock transfectants (Fig. 4 C). In contrast, clustering of the α5β1 integrin did not stimulate phosphorylation of these molecules in either the mock or β4 transfectants. Collectively, these data demonstrate the preferential ability of the α6β4 integrin to regulate the mTOR signaling pathway and, more importantly, the phosphorylation of 4E-BP1.

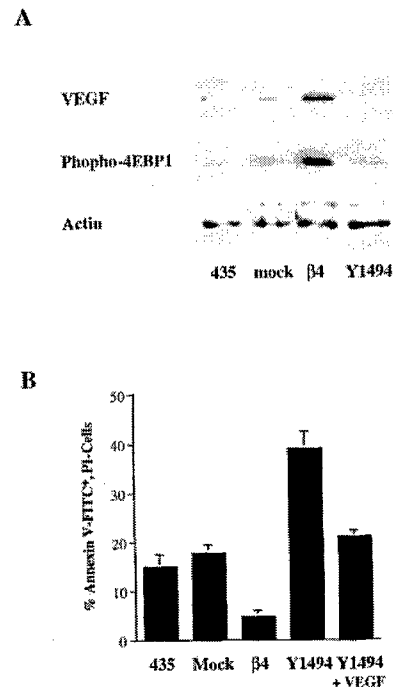


**Figure 5. Stimulation of 4E-BP1 phosphorylation, VEGF expression, and survival by the  $\alpha 6 \beta 4$  integrin requires PI-3K and mTOR.**

(A) MDA-MB-435  $\beta 4$  transfectants (clone 3A7) were incubated with either DMSO (–), the PI-3K inhibitor LY 294002 (10  $\mu$ M) (LY), or the mTOR-specific inhibitor rapamycin (50nM) (RPA) for 30 min and then incubated with either IgG or the  $\alpha 6$  integrin antibody 2B7 as described in the legend to Fig. 2. Extracts of these cells were immunoblotted for phospho-4E-BP1 (Ser65), VEGF, phospho-Erk (recognizing phosphorylated isoforms of ERK1 and ERK2), phospho-Akt (Ser 473), and total Akt. Similar data were obtained in three experiments. (B) MDA-MB-435  $\beta 4$  transfectants (clone 3A7) were maintained at low serum (0.5%) medium for 24 h in the presence of either rapamycin (50nM) (RPA), LY 294002 (10  $\mu$ M) (LY), or DMSO (–). Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V-FITC-positive, PI-negative cells. The data shown are mean values ( $\pm$  SD) of a representative experiment performed in triplicate.

To establish that PI-3K and mTOR are required for 4E-BP1 phosphorylation and VEGF expression, we performed the antibody clustering experiments in the presence of the PI-3K-specific inhibitor LY294002 and the mTOR-specific inhibitor rapamycin (Fig. 5). As shown in Fig. 5 A, both of these inhibitors blocked the  $\alpha 6 \beta 4$ -mediated induction of 4E-BP1 phosphorylation and VEGF expression. Although rapamycin did not block Akt phosphorylation, LY294002 did inhibit its phosphorylation, confirming that Akt acts upstream of mTOR and downstream of PI-3K (Fig. 5 A). These inhibitors did not block the phosphorylation of ERK1 and ERK2 (Fig. 5 A).

Finally, we investigated the importance of the mTOR pathway in survival, using rapamycin and LY294002. As shown in Fig. 5 B, rapamycin treatment increased the apop-

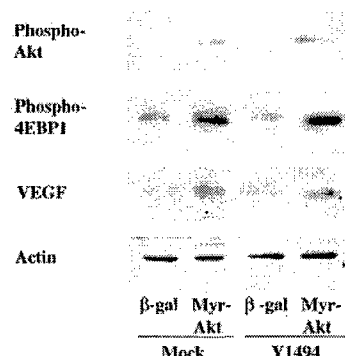


**Figure 6. Y1494 in the  $\beta 4$  cytoplasmic domain is required for  $\alpha 6 \beta 4$  stimulation of 4E-BP1 phosphorylation, VEGF expression, and survival.** (A) MDA-MB-435 parental cells (435), mock transfectants (clone 6D7), wild-type  $\beta 4$  transfectants (clone 3A7), and Y1494F mutant transfectants (clone E1h) were maintained in low serum (0.5% FBS) for 24 h. Extracts from these cells were analyzed by immunoblotting to assess the relative expression of VEGF and 4E-BP1 phosphorylation. The relative amount of actin was also determined as a control for protein loading. Similar results were obtained in three experiments. (B) Aliquots of the same cell populations described in A were assayed for the level of apoptosis after a 24-h incubation in low serum (0.5% FBS) medium. Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V-FITC-positive, PI-negative cells. The data shown are mean values ( $\pm$  SD) of three experiments performed in triplicate.

tosis of the MDA-MB-435/ $\beta 4$  transfectants fivefold and LY294002 treatment increased their apoptosis eightfold. These results indicate that the PI-3K–mTOR pathway is critical for the survival of these cells.

#### Identification of a specific tyrosine residue in the $\beta 4$ cytoplasmic domain required for $\alpha 6 \beta 4$ stimulation of 4E-BP1 phosphorylation and VEGF expression

Recently, a critical tyrosine residue (Y1494) was identified in the third fibronectin type III repeat of the  $\beta 4$  cytoplasmic domain, and this tyrosine was shown to be essential for activation of PI-3K by  $\alpha 6 \beta 4$  (Shaw, 2001). To assess the importance of Y1494 in 4E-BP1 phosphorylation and VEGF expression, stable subclones of MDA-MB-435 cells were generated that expressed  $\alpha 6 \beta 4$  containing a Y1494F mutation. As shown in Fig. 6 A, VEGF protein expression was barely detectable in these transfectants compared with the wild-type transfectants. Also, the steady-state level of 4E-BP1 phosphorylation was substantially lower in the Y1494F mutant transfectants than in the wild-type  $\beta 4$  transfectants. Interestingly, these mutant transfectants also exhibited an



**Figure 7. Expression of a constitutively active Akt construct mimics the effects of  $\alpha 6 \beta 4$  integrin expression and signaling.** MDA-MB-435 mock transfectants (clone 6D7) and Y1494F mutant transfectants (clone E1h) were infected with adenoviruses that expressed either  $\beta$ -galactosidase or Myr-Akt as described in the Materials and methods. Subsequently, the cells were incubated in low serum (0.5% FBS) medium for 24 h. Extracts of these cells were immunoblotted to assess the relative phosphorylation of Akt and 4E-BP1, as well as total expression of VEGF and actin.

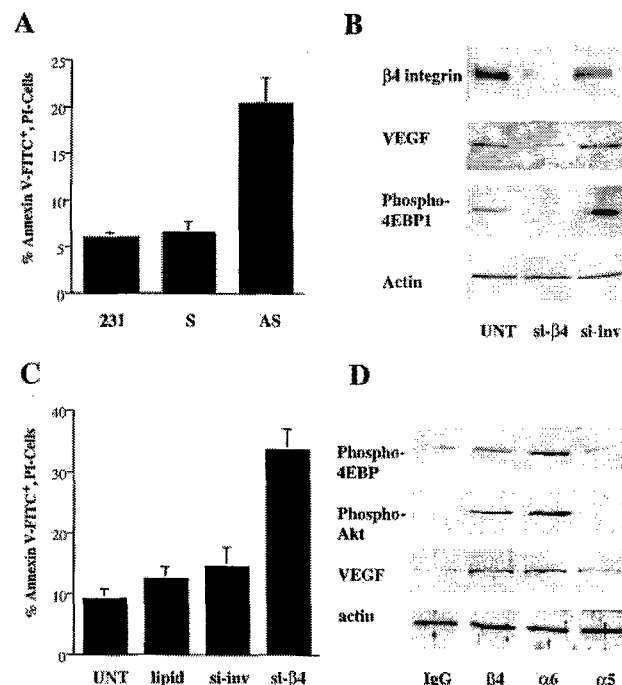
eightfold higher level of apoptosis than the wild-type  $\beta 4$  transfectants in response to serum deprivation (Fig. 6 B). The apoptosis of the mutant cells was reduced substantially by the addition of recombinant VEGF (Fig. 6 B), a result that substantiates the importance of VEGF in the survival of these cells. Together, these findings highlight the importance of the  $\beta 4$  cytoplasmic domain and PI-3K signaling in the regulation of VEGF expression and tumor cell survival.

#### Expression of constitutively active Akt stimulates 4E-BP1 phosphorylation and VEGF expression in the absence of $\alpha 6 \beta 4$ signaling

The hypothesis that activation of Akt is a major determinant for the stimulation of 4E-BP1 phosphorylation and VEGF expression was assessed by expressing a constitutively active Akt construct in MDA-MB-435 cells that are deficient in  $\alpha 6 \beta 4$  signaling. For this purpose, we used MDA-MB-435/mock transfectants that lack  $\alpha 6 \beta 4$  expression and the MDA-MB-435/ $\beta 4$  Y1494F transfectants, described above, which are deficient in  $\alpha 6 \beta 4$ -mediated activation of PI-3K. These cells were infected with adenoviruses that encoded either a myristoylated Akt (Myr-Akt) construct or  $\beta$ -galactosidase as a control. As shown in Fig. 7, expression of Myr-Akt stimulated 4E-BP1 phosphorylation and VEGF expression substantially in both populations of transfectants in comparison to cells that expressed  $\beta$ -galactosidase. This result indicates the critical importance of Akt activation by  $\alpha 6 \beta 4$  for stimulating VEGF expression.

#### $\alpha 6 \beta 4$ regulates 4E-BP1 phosphorylation, VEGF expression, and survival in carcinoma cells that express this integrin endogenously

Given that the data reported above are based on the exogenous expression of  $\alpha 6 \beta 4$  in  $\alpha 6 \beta 4$ -deficient carcinoma cells, we sought to extend our findings to cells that express this integrin endogenously, a pattern that is typical of most carcinoma cells. For this purpose, we used MDA-MB-231 breast carcinoma cells because they express the  $\alpha 6 \beta 4$  and  $\alpha 5 \beta 1$  in-



**Figure 8.  $\alpha 6 \beta 4$  regulates 4E-BP1 phosphorylation, VEGF expression, and survival in carcinoma cells that express this integrin endogenously.** (A) Parental MDA-MB-231 cells and cells transfected with antisense or sense VEGF oligonucleotides were maintained in low serum (0.5% FBS) medium for 24 h. Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V-FITC-positive, PI-negative cells. The data shown are mean values ( $\pm$  SD) of two separate experiments performed in triplicate. (B) MDA-MB-231 cells were left untreated (UNT) or were transfected with either an RNAi specific for the  $\beta 4$  integrin (si- $\beta 4$ ) or the corresponding inverted sequence (si-inv). After 72 h, the cells were placed in medium containing low serum (0.5% FBS) for an additional 24 h and then extracted. Extracts of these cells were immunoblotted as described in the legend to Fig. 4 to assess expression of  $\beta 4$  integrin, VEGF, and actin, as well as the phosphorylation of 4E-BP1. Similar results were observed in three independent trials. (C) Apoptosis was assessed in the same populations of cells and is reported as the percentage of annexin V-FITC-positive, PI-negative cells. The data shown are mean values ( $\pm$  SD) of three independent experiments performed in triplicate. (D) MDA-MB-231 cells were maintained in low serum (0.5% FBS) medium for 24 h and harvested by trypsin treatment. The suspended cells were incubated with integrin-specific antibodies ( $\beta 4$  integrin, A9;  $\alpha 6$  integrin, 2B7;  $\alpha 5$  integrin, Sam1) or IgG for 30 min in suspension and allowed to adhere on anti-IgG-coated plates for 30 min. Extracts of these cells were immunoblotted with phosphospecific antibodies to assess the relative phosphorylation of Akt and 4E-BP1, as well as with antibodies specific for VEGF and actin. Similar results were obtained in five experiments.

tegrins (Plopper et al., 1998; Mukhopadhyay et al., 1999; Saad et al., 2000). Initially, we confirmed that the survival of these cells is dependent on their expression of VEGF. As shown in Fig. 8 A, expression of a VEGF antisense oligonucleotide in these cells (Bachelder et al., 2001) resulted in an approximate fourfold increase in annexin V staining upon serum starvation compared with either untreated cells or cells that expressed the sense oligonucleotide.

To establish a role for  $\alpha 6 \beta 4$  in regulating VEGF expression and survival rigorously, we used a small interfering

RNA (RNAi) approach to inhibit  $\beta 4$  expression in MDA-231 cells. RNAis specific for the  $\beta 4$  subunit and the corresponding inverted sequence were designed and expressed in these cells by transfection. The cells were maintained in low serum (0.5%) for 24 h after transfection and then analyzed. As shown in Fig. 8 B, cells transfected with the RNAi specific for  $\beta 4$  exhibited a significant reduction in  $\beta 4$  expression in comparison with either untransfected cells or cells transfected with the inverted sequence. Importantly, the reduction in  $\beta 4$  expression by RNAi coincided with a marked reduction in 4E-BP1 phosphorylation and in the steady-state level of VEGF (Fig. 8 B), as well as an approximate threefold increase in annexin V staining (Fig. 8 C). These results link  $\alpha 6 \beta 4$  expression directly to 4E-BP1 phosphorylation, VEGF expression, and survival in a carcinoma cell line that expresses endogenous  $\alpha 6 \beta 4$ .

Subsequently, we performed antibody clustering experiments to substantiate the regulation of VEGF expression by  $\alpha 6 \beta 4$  (Fig. 8 D). Clustering of the  $\alpha 6 \beta 4$  integrin with either an  $\alpha 6$  integrin-specific antibody (mAb 2B7) or a  $\beta 4$  integrin-specific antibody (mAb A9) stimulated the phosphorylation of 4E-BP1 and Akt, and increased VEGF expression. In contrast, no induction of VEGF expression or stimulation of either 4E-BP1 or Akt phosphorylation was observed upon clustering with an  $\alpha 5$  integrin-specific antibody (mAb Sam1) or IgG.

## Discussion

This study establishes a novel mechanism by which integrins regulate growth factor expression. Specifically, our findings demonstrate the ability of a specific integrin ( $\alpha 6 \beta 4$ ), which has been implicated in carcinoma progression (Mercurio and Rabinovitz, 2001), to stimulate the translation of VEGF and sustain a VEGF autocrine loop that is essential for survival. More specifically, we define a signaling pathway regulated by  $\alpha 6 \beta 4$  that involves the preferential ability of this integrin to stimulate the phosphorylation of 4E-BP1 by activating the PI-3K-Akt pathway. As shown previously, this phosphorylation event dissociates 4E-BP1 from eIF-4E, enabling this key elongation factor to mediate the translation of VEGF and other functionally important molecules (De Benedetti and Harris, 1999; Gingras et al., 1999, 2001b; McKendrick et al., 1999). Moreover, the polysome analysis and antisense eIF-4E results we provide indicate that  $\alpha 6 \beta 4$  stimulation of VEGF translation is cap dependent and probably doesn't involve the internal ribosome entry sites that are present in the VEGF mRNA (Huez et al., 1998; van der Velden and Thomas, 1999). Our data extend earlier reports on the involvement of eIF-4E, VEGF, and  $\alpha 6 \beta 4$  in carcinoma progression by linking these molecules in a common signaling pathway that promotes tumor survival. Furthermore, they reveal a role for integrins in regulating growth factor expression by stimulating protein translation.

An important and novel aspect of our findings is that they add a new dimension to the understanding of how integrins promote cell survival. The widely accepted notion is that integrins, often in concert with growth factor receptors, activate specific signaling pathways that sustain survival (Taylor et al., 1999; Liu et al., 2000). We demonstrate here that the

survival function of integrins may not only be mediated by the activation of a key survival kinase such as Akt and the consequent effects of Akt on apoptotic signaling (Datta et al., 1999) but also by the Akt-dependent translation and expression of growth factors, such as VEGF, that promote survival in an autocrine, and possibly paracrine, fashion. In other terms, our results reveal that VEGF is a novel target of Akt signaling by integrins that is important for survival and distinct from known survival factors that are downstream of Akt, such as Bad (Datta et al., 1999). Importantly, our recent observation that VEGF stimulates the PI-3K-Akt pathway in carcinoma cells (Bachelder et al., 2001), in conjunction with our finding that  $\alpha 6 \beta 4$  signaling enhances VEGF expression, leads to the conclusion that integrin-mediated activation of PI-3K-Akt is amplified by integrin-stimulated VEGF expression. Moreover, we show that this amplification of PI-3K-Akt activity is important for carcinoma survival.

Although  $\alpha 6 \beta 4$  activates PI-3K in carcinoma cells (Gambaletta et al., 2000; Nguyen et al., 2000; Hintermann et al., 2001; Trusolino et al., 2001), no attempt had been made to link this signaling event with downstream effectors that regulate protein translation, namely mTOR and 4E-BP1. One reason that this possibility had not been explored is because a role for  $\alpha 6 \beta 4$  in regulating either protein translation or growth factor expression was not obvious. In fact, almost all of the functional studies on  $\alpha 6 \beta 4$  in carcinoma cells have focused on its role in promoting migration and invasion, and on the mechanism by which  $\alpha 6 \beta 4$ -mediated signaling influences these processes (Mercurio, 1990; Shaw et al., 1997; Gambaletta et al., 2000; Trusolino et al., 2001). Our motivation to examine a possible connection between  $\alpha 6 \beta 4$  and VEGF translation was based on our interest in understanding the mechanisms by which these molecules promote the survival of carcinoma cells. Indeed, our results establish a role for  $\alpha 6 \beta 4$  in survival signaling by regulating VEGF translation, but the implications of these findings are more widespread. For example, recent studies that have argued that  $\alpha 6 \beta 4$  is necessary for growth factor receptor (erbB2, c-met) activation of PI-3K (Gambaletta et al., 2000; Trusolino et al., 2001) raise the interesting possibility of an intimate functional association among specific growth factor receptors,  $\alpha 6 \beta 4$ , VEGF, and PI-3K, all of which have been implicated in tumor progression.

Surprisingly, few studies have examined the role of integrin signaling in regulating protein translation (e.g., Pabla et al., 1999). Indeed, there has been much more interest in defining the contribution of integrins to transcription. The ability of integrins to regulate translation, however, provides a mechanism for altering cell function rapidly, by increasing the expression of specific proteins. This possibility is exemplified by our finding that ligation of the  $\alpha 6 \beta 4$  integrin resulted in a significant increase in VEGF protein within 60 min (Fig. 2 C). Given the fact that eIF-4E is rate limiting for the translation of proteins involved in growth control and other critical cell functions (De Benedetti and Harris, 1999), the hypothesis can be formulated that integrin-mediated regulation of translation contributes to the ability of cells to alter their behavior rapidly in response to changes in their microenvironment. This hypothesis is particularly relevant to our interest in the regulation of VEGF expression. Al-

though much of the work in this area has focused on the ability of hypoxia to stimulate VEGF transcription and increase the stability of VEGF mRNA (von Marschall et al., 2001), it has become apparent that translational control is also important (Kevil et al., 1996; De Benedetti and Harris, 1999). Moreover, our recent finding that VEGF is essential for the survival of breast carcinoma cells in normoxia substantiates the functional importance of integrin-mediated regulation of VEGF expression (Bachelder et al., 2001).

The fact that our data implicate eIF-4E in tumor cell survival is of considerable interest because recent studies have revealed an important role for this elongation factor in cancer (DeFatta et al., 1999, 2000; Ernst-Stecken, 2000; Berkel et al., 2001). Overexpression of this factor in NIH3T3 cells, as well as other "normal" cells, stimulates division and can induce their transformation (Fukuchi-Shimogori et al., 1997). These findings are consistent with the reports that the expression of eIF-4E is elevated in solid tumors compared with normal tissue (De Benedetti and Harris, 1999). Moreover, hypoxia, a pathophysiological stress that provides a selective pressure for the survival of aggressive tumor cells, enhances eIF-4E expression (DeFatta et al., 1999). Together, these observations highlight an important role for translational control in human cancer. This role is substantiated by the fact that eIF-4E controls the translation of not only VEGF but also other molecules that influence tumor growth and survival such as c-Myc, cyclin D1, and FGF-2 (De Benedetti and Harris, 1999). From our perspective, we are intrigued by the reports that the  $\alpha 6 \beta 4$  integrin is associated with the progression of many solid tumors, and its expression has been correlated with a poorer prognosis in patients with some of these tumors (Mercurio and Rabinovitz, 2001). Our finding that  $\alpha 6 \beta 4$  can induce the translational function of eIF-4E by regulating the phosphorylation of 4E-BP1 provides one mechanism to account for the role of this integrin in cancer.

## Materials and methods

### Cells

MDA-MB-231 and MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University. They were grown in low glucose DME containing 10% FBS, 1% penicillin-streptomycin, and 25 mM Hepes. For inhibitor experiments, cells were harvested by trypsinization and suspended cells were incubated with rapamycin (Calbiochem) at 100 nM or LY 294002 (Calbiochem) at 10  $\mu$ M on ice for 30 min before they were plated at 37°C for the experiment.

The generation of MDA-MB-435 subclones expressing the  $\alpha 6 \beta 4$  integrin has been described previously (Shaw et al., 1997). Tyrosine residue 1494 in the  $\beta 4$  subunit was mutated to a phenylalanine residue using the Quickchange site-directed mutagenesis kit (Stratagene), and stable subclones of MDA-MB-435 cells that expressed  $\alpha 6 \beta 4$  containing this mutant  $\beta 4$  subunit were generated (Shaw, 2001).

For adenoviral infection, cells were grown in DME containing 10% FBS until they reached 50% confluency. At this point, the culture medium was changed to DME containing 0.5% FBS. Viral dilutions were prepared from purified viral stocks in DME containing 0.5% FBS and the cells were infected for 4 h. At the end of the infection period, the virus-containing medium was removed and the cells were washed once with PBS, and incubated for an additional 12 h in DME containing 10% FBS.

### Apoptosis assays

To induce apoptosis, cells were incubated in DME containing 0.5% FBS for 24 h at 37°C. Subsequently, both adherent and nonadherent cells were harvested and their level of apoptosis was assessed using annexin V-FITC. In brief, cells were washed once with serum-containing medium, once

with PBS, once with annexin V-FITC buffer (10 mM Hepes-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ), and then incubated for 15 min at room temperature with 5  $\mu$ g/ml annexin V-FITC (Biosource International). After washing once with annexin V buffer, the samples were resuspended in the same buffer and analyzed by flow cytometry. Immediately before the analysis, 5  $\mu$ g/ml propidium iodide (PI) (Biosource International) was added to distinguish apoptotic cells from necrotic cells.

### Quantitative real-time PCR

Quantitative analysis of VEGF mRNA expression was performed by real-time PCR using an ABI Prism 7700 sequence detection system (Perkin-Elmer) and SYBR green master mix kit as described previously (Bachelder et al., 2001). Sequences of primers and probes were as follows: VEGF forward primer, 5'-GAAGTGGTGAAGTTCATGGATGCTA-3'; VEGF reverse primer, 5'-TGGAAGATGTCCACCAGGCT-3'; VEGF probe, 5'-TET/AGCGCAGCTACTGCCATCCAATCG/TAM-3';  $\beta$ -actin forward primer, 5'-TCACCATGGATGATGATATCGC-3';  $\beta$ -actin reverse primer, 5'-AAGC-CGGCCTTGACAT-3'; and  $\beta$ -actin probe, 5'-FAM/CGCTCGTCGTCGCA-CAACGGCT/TAM-3'. The data obtained are presented as the mean ratio of VEGF to  $\beta$ -actin mRNA ( $\pm$  SD) obtained from triplicate samples.

### VEGF antisense oligonucleotide experiments

A VEGF antisense 2'-O-methyl phosphorothioate oligodeoxynucleotide (5'-CACCCAAGACAGCAGAA-3') and a sense 2'-O-methyl phosphorothioate oligodeoxynucleotide (5'-CTTCTGCTGCTCTGGGTG) (provided by Greg Robinson, Children's Hospital, Boston, MA) were used to transfect MDA-MB-435  $\beta 4$  transfectants at a concentration of 0.3  $\mu$ M in the presence of lipofectin reagent (2  $\mu$ g/ml; GIBCO BRL). The experimental details for this approach have been described previously (Bachelder et al., 2001). In addition, the same protocol was used to express antisense and sense eIF-4E oligonucleotides, which were gifts from Arigo De Benedetti (Louisiana State University, Shreveport, LA) (DeFatta et al., 2000).

### RNAi experiments

An RNAi specific for the  $\beta 4$  integrin subunit (GAGCUGCACGGAGUGUGUC) as well as the inverted sequence (CUGUGAGGACGUCGAG) were designed and synthesized by Dharmacon, Inc. MDA-231 cells at 30% confluency were transfected with 300 pmoles of RNAi using TKO lipids (Mirus). Subsequently, the cells were maintained in complete medium for 72 h and in medium containing 0.5% FBS for an additional 24 h before analysis.

### Polysome analysis

Cells ( $3 \times 10^7$ ) were maintained in medium containing low serum (0.5% FBS) for 24 h and then pretreated with 100  $\mu$ g/ml cycloheximide (Calbiochem) for 15 min at 37°C before being harvested. After washing once with PBS containing 100  $\mu$ g/ml cycloheximide, the cells were resuspended in 0.5 ml of a modified U+S buffer (Davies and Abe, 1995). This buffer was composed of 200 mM Tris-HCl (pH 8.8), 25 mM  $\text{MgCl}_2$ , 5 mM EGTA (pH 8.0), 150 mM KCl, 10  $\mu$ g/ml heparin, 5 mM DTT, 1% sodium deoxycholate, 2% polyoxyethylene 10-tridecyl ester, 100  $\mu$ g/ml cycloheximide, and 200 mM sucrose. Ribonuclease inhibitor (Amersham Biosciences) was added to a final concentration of 0.5 U/ $\mu$ l. Cells were homogenized with 20–25 strokes in a Kontes tissue homogenizer, followed by centrifugation for 5 min at 14,000 g. The supernatant was collected and frozen at  $-80^\circ\text{C}$  until further use. Sucrose gradients (15–50%, wt/wt) were layered with 300  $\mu$ l of cleared cell extract, which was then centrifuged at 160,000 g for 2 h. Fractions (0.75–0.375 ml) were withdrawn from the top of the gradient and monitored for absorbency at 254 nm using an ISCO syringe pump with UV-6 detector. Total RNA from the sucrose gradient fractions was extracted using Trizol LS (Life Technologies) according to the manufacturer's instructions. Quantitative real-time PCR was used to measure the VEGF mRNA level in each fraction as described above.

### Integrin signaling experiments

Cells were harvested by trypsin treatment and washed twice with DME containing 25 mM Hepes and 0.1% BSA. After washing, the cells were resuspended in the same buffer at a concentration of  $2 \times 10^6$  cells/ml and incubated for 30 min with integrin-specific antibodies (4  $\mu$ g/ml) or with either mouse or rat IgG (4  $\mu$ g/ml). The cells were washed once, resuspended in the same buffer, and added to plates that had been coated overnight with either the anti-mouse or rat IgG. After a 60-min incubation at 37°C, the cells that had attached to integrin-specific antibodies were washed twice with cold PBS and solubilized at 4°C for 10 min using RIPA buffer (20 mM Tris buffer, pH 7.4, containing 0.14 M NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, 5  $\mu$ g/ml aprotinin,



pepstatin, and leupeptin). The IgG-treated cells were harvested by centrifugation and solubilized with RIPA buffer.

### Protein analysis

Aliquots of cell extracts containing equivalent amounts of protein were solubilized using 5× sample buffer containing 100 mM DTT and then incubated at 100°C for 15 min. These extracts were resolved by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked for 1 h using a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 0.05% Tween-20 (TBST), and 5% (wt/vol) Carnation dry milk. The filters were incubated overnight in the same buffer containing antibodies specific for p70S6K, 4EBP antibodies (Santa Cruz Biotechnology, Inc.), actin (ICN Biomedicals), and VEGF (clone 618, provided by Donald Senger, Beth Israel Deaconess Medical Center). After three, 10-min washes in TBST, the filters were incubated for 1 h in blocking buffer containing HRP-conjugated secondary antibodies. After three 10-min washes in TBST, proteins were detected by ECL (Pierce Chemical Co.).

For immunoblots involving phosphospecific antibodies, the filters were blocked for 1 h using a 10 mM Tris buffer, pH 7.5, containing 0.5 M NaCl, 0.1% Tween-20, and 2% (wt/vol) BSA. The filters were washed briefly and then incubated overnight at 4°C in the same blocking buffer containing antibodies specific for phospho-p70S6K (Thr-389; Cell Signaling Technology), phospho-4E-BP1 (Ser-65; Cell Signaling Technology), phospho-Erk (E10; Cell Signaling Technology), and phospho-Akt (Ser-473 clone 4E2; Cell Signaling Technology). After washing, the filters were incubated for 1 h in blocking buffer containing HRP-conjugated secondary antibody and the proteins were detected by ECL.

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## References

- Akiri, G., D. Nahari, Y. Finkelstein, S.Y. Le, O. Elroy-Stein, and B.Z. Levi. 1998. Regulation of vascular endothelial growth factor (VEGF) expression is mediated by internal initiation of translation and alternative initiation of transcription. *Oncogene*. 17:227–236.
- Bachelder, R.E., A. Marchetti, R. Falcioni, S. Soddu, and A.M. Mercurio. 1999a. Activation of p53 function in carcinoma cells by the  $\alpha 6\beta 4$  integrin. *J. Biol. Chem.* 274:20733–20737.
- Bachelder, R.E., M.J. Ribick, A. Marchetti, R. Falcioni, S. Soddu, K.R. Davis, and A.M. Mercurio. 1999b. p53 inhibits  $\alpha 6\beta 4$  integrin survival signaling by promoting the caspase 3-dependent cleavage of AKT/PKB. *J. Cell Biol.* 147:1063–1072.
- Bachelder, R.E., A. Crago, J. Chung, M.A. Wendt, L.M. Shaw, G. Robinson, and A.M. Mercurio. 2001. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res.* 61:5736–5740.
- Berkel, H.J., E.A. Turbat-Herrera, R. Shi, and A. de Benedetti. 2001. Expression of the translation initiation factor eIF4E in the polyp-cancer sequence in the colon. *Cancer Epidemiol. Biomarkers Prev.* 10:663–666.
- Brown, L.F., A.J. Guidi, S.J. Schnitt, L. Van De Water, M.L. Iruela-Arispe, T.K. Yeo, K. Tognazzi, and H.F. Dvorak. 1999. Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. *Clin. Cancer Res.* 5:1041–1056.
- Datta, S.R., A. Brunet, and M.E. Greenberg. 1999. Cellular survival: a play in three Acts. *Genes Dev.* 13:2905–2927.
- Davies, E., and S. Abe. 1995. Methods for isolation and analysis of polyribosomes. *Methods Cell Biol.* 50:209–222.
- De Benedetti, A., and A.L. Harris. 1999. eIF4E expression in tumors: its possible role in progression of malignancies. *Int. J. Biochem. Cell Biol.* 31:59–72.
- DeFatta, R.J., E.A. Turbat-Herrera, B.D. Li, W. Anderson, and A. De Benedetti. 1999. Elevated expression of eIF4E in confined early breast cancer lesions: possible role of hypoxia. *Int. J. Cancer.* 80:516–522.
- DeFatta, R.J., C.A. Nathan, and A. De Benedetti. 2000. Antisense RNA to eIF4E suppresses oncogenic properties of a head and neck squamous cell carcinoma cell line. *Laryngoscope.* 110:928–933.
- Dennis, P.B., S. Fumagalli, and G. Thomas. 1999. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Curr. Opin. Genet. Dev.* 9:49–54.
- Dvorak, H.F., J.A. Nagy, D. Feng, L.F. Brown, and A.M. Dvorak. 1999. Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. *Curr. Top. Microbiol. Immunol.* 237:97–132.
- Ernst-Stecken, A. 2000. The molecular marker eIF4E in the surgical margins of the resection preparations of head-neck tumors as a prognostic factor. *Strahlenther. Onkol.* 176:383–384.
- Fearon, E.R. 1999. Cancer progression. *Curr. Biol.* 9:R873–R875.
- Fukuchi-Shimogori, T., I. Ishii, K. Kashiwagi, H. Mashiba, H. Ekimoto, and K. Igarashi. 1997. Malignant transformation by overproduction of translation initiation factor eIF4G. *Cancer Res.* 57:5041–5044.
- Gambaletti, D., A. Marchetti, L. Benedetti, A.M. Mercurio, A. Sacchi, and R. Falcioni. 2000. Cooperative signaling between  $\alpha 6\beta 4$  integrin and ErbB-2 receptor is required to promote phosphatidylinositol 3-kinase-dependent invasion. *J. Biol. Chem.* 275:10604–10610.
- Gingras, A.C., S.P. Gygi, B. Raught, R.D. Polakiewicz, R.T. Abraham, M.F. Hoekstra, R. Aebersold, and N. Sonenberg. 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev.* 13:1422–1437.
- Gingras, A.C., B. Raught, S.P. Gygi, A. Niedzwiecka, M. Miron, S.K. Burley, R.D. Polakiewicz, A. Wyslouche-Cieszyńska, R. Aebersold, and N. Sonenberg. 2001a. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev.* 15:2852–2864.
- Gingras, A.C., B. Raught, and N. Sonenberg. 2001b. Regulation of translation initiation by FRAP/mTOR. *Genes Dev.* 15:807–826.
- Hanahan, D., and J. Folkman. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell.* 86:353–364.
- Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell.* 100:57–70.
- Hintermann, E., M. Bilban, A. Sharabi, and V. Quaranta. 2001. Inhibitory role of  $\alpha 6\beta 4$ -associated erbB-2 and phosphoinositide 3-kinase in keratinocyte haptotactic migration dependent on  $\alpha 3\beta 1$  integrin. *J. Cell Biol.* 153:465–478.
- Huez, I., L. Creancier, S. Audigier, M.C. Gensac, A.C. Prats, and H. Prats. 1998. Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. *Mol. Cell Biol.* 18:6178–6190.
- Kevil, C.G., A. De Benedetti, D.K. Payne, L.L. Coe, F.S. Laroux, and J.S. Alexander. 1996. Translational regulation of vascular permeability factor by eukaryotic initiation factor 4E: implications for tumor angiogenesis. *Int. J. Cancer.* 65:785–790.
- Liu, W., S.A. Ahmad, N. Reinmuth, R.M. Shaheen, Y.D. Jung, F. Fan, and L.M. Ellis. 2000. Endothelial cell survival and apoptosis in the tumor vasculature. *Apoptosis.* 5:323–328.
- McKendrick, L., V.M. Pain, and S.J. Morley. 1999. Translation initiation factor 4E. *Int. J. Biochem. Cell Biol.* 31:31–35.
- Mercurio, A.M. 1990. Laminin: multiple forms, multiple receptors. *Curr. Opin. Cell Biol.* 2:845–849.
- Mercurio, A.M., and I. Rabinovitz. 2001. Towards a mechanistic understanding of tumor invasion: lessons from the  $\alpha 6\beta 4$  integrin. *Semin. Cancer Biol.* 11:129–141.
- Mukhopadhyay, R., R.L. Thériault, and J.E. Price. 1999. Increased levels of  $\alpha 6\beta 4$  integrins are associated with the metastatic phenotype of human breast cancer cells. *Clin. Exp. Metastasis.* 17:325–332.
- Nabors, L.B., G.Y. Gillespie, L. Harkins, and P.H. King. 2001. HuR, a RNA stability factor, is expressed in malignant brain tumors and binds to adenine- and uridine-rich elements within the 3' untranslated regions of cytokine and angiogenic factor mRNAs. *Cancer Res.* 61:2154–2161.
- Nguyen, B.P., S.G. Gil, and W.G. Carter. 2000. Deposition of laminin 5 by keratinocytes regulates integrin adhesion and signaling. *J. Biol. Chem.* 275:31896–31907.
- Pabla, R., A.S. Weyrich, D.A. Dixon, P.F. Bray, T.M. McIntyre, S.M. Prescott, and G.A. Zimmerman. 1999. Integrin-dependent control of translation: engagement of integrin  $\alpha 11\beta 3$  regulates synthesis of proteins in activated human platelets. *J. Cell Biol.* 144:175–184.
- Plopper, G.E., S.Z. Domanico, V. Cirulli, W.B. Kiosses, and V. Quaranta. 1998. Migration of breast epithelial cells on laminin-5: differential role of integrins in normal and transformed cell types. *Breast Cancer Res. Treat.* 51:57–69.
- Raught, B., and A.C. Gingras. 1999. eIF4E activity is regulated at multiple levels.



- Int. J. Biochem. Cell Biol.* 31:43–57.
- Saad, S., L.J. Bendall, A. James, D.J. Gottlieb, and K.F. Bradstock. 2000. Induction of matrix metalloproteinases MMP-1 and MMP-2 by co-culture of breast cancer cells and bone marrow fibroblasts. *Breast Cancer Res. Treat.* 63:105–115.
- Schmelzle, T., and M.N. Hall. 2000. TOR, a central controller of cell growth. *Cell* 103:253–262.
- Scotlandi, K., S. Benini, M. Sarti, M. Serra, P.L. Lollini, D. Maurici, P. Picci, M.C. Manara, and N. Baldini. 1996. Insulin-like growth factor I receptor-mediated circuit in Ewing's sarcoma/peripheral neuroectodermal tumor: a possible therapeutic target. *Cancer Res.* 56:4570–4574.
- Sekulic, A., C.C. Hudson, J.L. Homme, P. Yin, D.M. Otterness, L.M. Karnitz, and R.T. Abraham. 2000. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res.* 60:3504–3513.
- Shaw, L.M. 2001. Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the  $\alpha 6 \beta 4$  integrin-dependent activation of phosphoinositide 3-OH kinase and promotion of invasion. *Mol. Cell. Biol.* 21:5082–5093.
- Shaw, L.M., I. Rabinovitz, H.H. Wang, A. Toker, and A.M. Mercurio. 1997. Activation of phosphoinositide 3-OH kinase by the  $\alpha 6 \beta 4$  integrin promotes carcinoma invasion. *Cell* 91:949–960.
- Shweiki, D., A. Itin, D. Soffer, and E. Keshet. 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843–845.
- Stein, I., A. Itin, P. Einat, R. Skalter, Z. Grossman, and E. Keshet. 1998. Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. *Mol. Cell. Biol.* 18:3112–3119.
- Taylor, S.T., J.A. Hickman, and C. Dive. 1999. Survival signals within the tumour microenvironment suppress drug-induced apoptosis: lessons learned from B lymphomas. *Endocr. Relat. Cancer* 6:21–23.
- Tokunou, M., T. Niki, K. Eguchi, S. Iba, H. Tsuda, T. Yamada, Y. Matsuno, H. Kondo, Y. Saitoh, H. Imamura, and S. Hirohashi. 2001. c-MET expression in myofibroblasts: role in autocrine activation and prognostic significance in lung adenocarcinoma. *Am. J. Pathol.* 158:1451–1463.
- Trusolino, L., A. Bertotti, and P.M. Comoglio. 2001. A signaling adapter function for  $\alpha 6 \beta 4$  integrin in the control of HGF-dependent invasive growth. *Cell* 107:643–654.
- van der Velden, A.W., and A.A. Thomas. 1999. The role of the 5' untranslated region of an mRNA in translation regulation during development. *Int. J. Biochem. Cell Biol.* 31:87–106.
- von Marschall, Z., T. Cramer, M. Hocker, G. Finkenzeller, B. Wiedenmann, and S. Rosewicz. 2001. Dual mechanism of vascular endothelial growth factor up-regulation by hypoxia in human hepatocellular carcinoma. *Gut* 48:87–96.
- Wong, A.S., S.L. Pelech, M.M. Woo, G. Yim, B. Rosen, T. Ehlen, P.C. Leung, and N. Auersperg. 2001. Coexpression of hepatocyte growth factor-Met: an early step in ovarian carcinogenesis? *Oncogene* 20:1318–1328.